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STUDIES RELATIVE TO THE APPARENT CLOSE RELATIONSHIP BETWEEN BACT. PERTUSSIS AND B. BRONCHISEPTICUS[1]

II. COMPLEMENT FIXATION TESTS

N. S. FERRY AND H. C. KLIX

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In a previous article (Ferry and Noble, 1918) we have described the cultural, agglutination and absorption reactions between *Bact. pertussis* and *B. bronchisepticus* and have shown that, while the two organisms are distinct, they are apparently somewhat closely related. The most striking characteristics of the organisms, according to the serological reactions, were shown to be the ability of *B. bronchisepticus* to produce an immune serum that would agglutinate both the *B. bronchisepticus* and *Bact. pertussis* antigens and the ability of *Bact. pertussis* to produce an immune serum that would agglutinate only the homologous antigen. The absorption reaction showed that the *B. bronchisepticus* antigen would absorb from the anti-bronchisepticus serum (a serum that contained agglutinins for both organisms) only the *B. bronchisepticus* agglutinin (the major agglutinin) leaving intact the agglutinin for *Bact. pertussis* (the minor agglutinin). This minor agglutinin could only be absorbed by the *Bact. pertussis* antigen. This type of an agglutinin was termed by the authors a "transitive" agglutinin.

The present investigation was undertaken to confirm the work of the previous paper through complement fixation tests and to determine, if possible, the value of this test in differentiating between the two organisms.

Strains used. At first a large number of strains of each organism were used, the same strains as those worked with in the pre-

[1] Presented at Eighteenth Annual Meeting of the Society of American Bacteriologists, New Haven, Conn., December 27–29, 1916.

vious experiments already described, but as it was found that all strains of the same organism gave similar reactions it was deemed advisable to cut down the number to three of each in order to save time. Of the *B. bronchisepticus*, no. 36 (dog), no. 123 (monkey) and human strains were tested; and of *Bact. pertussis*, no. 0363 (Bordet), no. 109 and no. 248 (Povitzky).

Technic. For the volume of the complement fixation tests it was found more satisfactory to use 2 cc. than 5 cc. as advised for the Wassermann test or 0.5 cc. suggested by Olmstead and Povitzky in a serological comparison of the Bordet-Gengou bacillus and hemoglobinophilic bacilli. The hemolytic system was composed of sheep cells in a 2 per cent suspension, guinea-pig complement in a 1 to 10 dilution and rabbit amboceptor in 1 to 1500 dilution. Complement titration was made by using 0.1 cc. of amboceptor and varying amounts of complement.

In determining the relationship of the various strains two units of both amboceptor and complement were employed. All titrations were incubated one hour before and one hour after the addition of the sensitized cells, at 32°C. The dilution was chosen in which complete hemolysis was produced, readings being made at the end of the hour's incubation. All serum was inactivated by heating in water bath one half hour at 56°C.

The antigen was titrated by mixing it in varying amounts with one unit of the hemolytic system.

Preparation of antigen. After trying out several methods of antigen preparation it was finally determined that filtered autolysates gave the most stable and satisfactory products.

The antigens were prepared as follows: The organisms were grown on agar for forty-eight hours at 37.5°C., then taken off and suspended in distilled water and shaken for forty-eight hours in a mechanical shaker. This suspension was then heated at 56°C. for one-half hour, incubated twelve hours, after which enough sodium chloride and formalin was added to make an 0.85 per cent and 0.5 per cent solution respectively. Filtration was carried on through asbestos.

Preparation of immune serum. The same serums were used for this work as for the previous experiments, a description of which has already been given.

The results of the complement fixation tests may be seen in the following table:

ANTIGENS	ANTISERUMS					
	B. bronchisepticus (dog) no. 36	B. bronchisepticus (monkey) no. 123	B. bronchisepticus (human)	Bact. pertussis no. 0363	Bact. pertussis no. 109	Bact. pertussis no. 248
B. bronchisepticus (dog) no. 123	+	+	+	−	−	−
B. bronchisepticus (monkey) no. 123	+	+	+	+	+	+
B. bronchisepticus (human)	+	+	+	+	+	+
Bact. pertussis no. 0363	+	+	+	+	+	+
Bact. pertussis no. 109	+	+	+	+	+	+
Bact. pertussis no. 248	+	+	+	+	+	+

+ Denotes complete inhibition of hemolysis.
− Denotes incomplete or no inhibition of hemolysis.

It was found in a large majority of the tests as represented in the chart, which is a composite, that the *B. bronchisepticus* immune serum bound the complement in the presence of both the bronchisepticus and pertussis antigens, while the *B. pertussis* immune serum bound the complement in the presence of the homologous antigen and also the human and monkey strains of *B. bronchisepticus*. It did not bind the complement in the presence of a dog strain of *B. bronchisepticus*.

SUMMARY

1. *B. bronchisepticus* immune serum bound the complement in the presence of both *B. bronchisepticus* and *Bact. pertussis* antigen.

2. *Bact. pertussis* immune serum bound the complement in the presence of *Bact. pertussis* antigen and *B. bronchisepticus* antigen of both human and monkey origin but not of dog origin.

3. The complement fixation test is not a reliable method of differentiating between the two organisms in question.

4. Bacterial autolysates were found to be the most stable and satisfactory antigens.

5. The complement fixation test was found to corroborate, in most respects, the agglutinin reactions reported in a previous paper.

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THE OCCURRENCE OF DIFFERENT TYPES OF THE COLON-AEROGENES GROUP IN WATER[1]

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Recent work on the colon-aerogenes group of bacteria (Levine, 1916; Rogers, Clark and Davis, 1914; Rogers, Clark and Evans, 1914 and 1915; Winslow and Kligler, 1916) has confirmed and amplified the view held by most bacteriologists that the group includes two distinct types or species represented by *B. coli* and *B. aerogenes*. Although the distinction between the two types has not been very clearly defined, the former has been considered as the predominant organism of the feces of warm blooded animals.

The work cited has demonstrated that there is a real difference between the two colon types and that this difference can be detected by certain simple laboratory tests.

The *B. coli* type is characterized by the production of almost exactly equal volumes of hydrogen and carbon dioxide in the anaerobic fermentation of glucose, the failure to give the Voges-Proskauer reaction, the formation of indol from tryptophane and the comparatively low fermentative ability of a greater part of the cultures.

The *B. aerogenes* type, on the other hand produces an excess of carbon dioxide over hydrogen in the anaerobic fermentation

[1] Published by permission of the Secretary of Agriculture.

The writer wishes to acknowledge his indebtedness to his colleagues, particularly to Dr. William M. Clark for assistance and for many suggestions.

Since this paper was written, the writer has had the privilege of reading two manuscripts by Winslow and Cohen. They cover much the same ground as this paper and the results and conclusions drawn are substantially in accord with those given here.

of glucose, gives a positive Voges-Proskauer reaction, usually fails to form indol from tryptophane and ferments many carbohydrates and alcohols. The tendency to produce rapidly a hydrogen ion concentration which will inhibit alkali formation and consequently any subsequent reduction of the acidity has been made use of by Clark (1915) to distinguish the *B. coli* type from *B. aerogenes* by the methyl red test.

The *B. coli* type is found abundantly in bovine feces while the *B. aerogenes* type is very rare. In human feces the latter type is more common, but apparently subject to considerable variation. These results agree with the conclusions of some of the earlier workers, particularly Clemesha (1912) who states that sucrose +, dulcite −, adonite −, inuline −, V. and P. + organisms are rare in feces (about 1 to 2 per cent). These characters agree with those of the typical high ratio or *B. aerogenes* cultures found in feces.

The work in our laboratories on the colon-aerogenes bacteria of human feces also shows a possible line of demarcation between two varieties of the *B. aerogenes* type. All of the *B. aerogenes* cultures isolated from human feces fermented adonite. Many formed indol, 60 per cent fermented starch and in other ways showed a higher fermentative ability than *B. aerogenes* cultures from other sources.

On the other hand the collection of cultures from grains, consisting very largely of *B. aerogenes* cultures, contained very few adonite fermenters. Those that did ferment adonite agreed very closely with the adonite fermenters of feces while the non fermenters were less active in the formation of indol and in the fermentation of sucrose, raffinose, starch, mannite and glycerine. Disregarding, for the present, any question of the value of the differences enumerated for purposes of classification we believe that it is safe to use them as an indication of the original source of the culture.

It does not necessarily follow that the only source of *B. coli* or the adonite fermenting *B. aerogenes* is the intestine of warm blooded animals but the relative frequency with which they occur there makes it highly probable that any culture with these characters, wherever found, came originally from this habitat.

The value from a sanitary standpoint of an ability to distinguish between different types of colon bacilli rests on the answer which we can give to certain questions. First we are concerned with possibilities of any type of colon bacillus existing normally outside of the digestive tract of animals. If a colon culture of any kind is isolated from water does it necessarily indicate fecal contamination or must we distinguish between those of fecal and those of nonfecal origin?

Secondly what is the fate of the fecal colon bacillus in water? Does it multiply or decrease rapidly? Does the typical fecal colon bacillus become attenuated so that its characters change and it can no longer be recognized?

The first question is partially answered by the results of our comparison of the characters of fecal colon with those from other sources, particularly from grains. The marked variation of the grain cultures from the fecal types indicates that water may receive bacteria of the colon-aerogenes group from other than fecal sources.

Some light has been thrown on the second problem by the observations of Clemesha (1912). He found that the different types of colon did not decrease in water at an equal rate and that in some cases there was an actual increase.

The characters used by Clemesha differ somewhat from those which we have used and only rough comparisons can be made. We have studied this problem by observations on water held in bottles, and on cultures in parchment sacs in running water, by watching the progressive changes in streams and by examination of individual samples from various sources.

While it is realized that the results are not sufficiently comprehensive to permit any positive conclusions it is hoped that they may present something of value.

CHANGES IN SAMPLES HELD IN BOTTLES

In considering bacterial changes in water samples held in bottles, one should remember that the conditions are not necessarily comparable with those obtained in streams or reservoirs.

The concentrations, both of food-stuffs and products of metabolism, are likely to be much greater in the bottle and the results may vary accordingly.

A number of bottles containing 100 cc. of sterile water to which a loopful of human feces had been added were left in a 20° incubator for several months.

These bottles were used for another purpose and from the earlier plates made only a few cultures were isolated. Later a total colon count was made and a considerable number of cultures isolated. In table 1 are given the results from a bottle which was held at 20° for 278 days.

TABLE 1

Changes in colon content in water held at 20°

AGE	COLON GROUP PER CUBIC CENTIMETER	CULTURES ISOLATED		P_H LIMIT OF B. COLI CULTURES						INDOL BY B. COLI CULTURES	
		B. aerogenes	B. coli	4.8	4.9	5.0	5.2	5.4	5.6	+	−
days											
0		0	3	1	2					3	0
17		1	8	2	5	1				8	0
31	4,700,000	1	6	2	3	1				5	0
47	2,100,000	0	9			9				9	0
78	3,530,000	0	12			11	1			11	1
109	2,530,000	4	46								
137	2,090,000	7	58								
160		8	32				23	9		24	1
199	174,000	11	31	2		26	1	1	1	23	8
278	38,750	39	1				1				

The total number of colon forms decreased slowly in this period. At first there was a great preponderance of *B. coli* over *B. aerogenes*. Even after 100 days there were ten times as many *B. coli* as *B. aerogenes*. But this relation was gradually changed until at 278 days there was only one *B. coli* in the 40 cultures isolated.

Another change which may be of some significance is found in the apparent decrease in limiting hydrogen ion concentration reached by the *B. coli* cultures. The P_H value of 5.0 to 4.8 observed in the first cultures isolated is normal for freshly iso-

lated *B. coli* cultures. In the *B. coli* cultures isolated after they had been exposed for a long time to the unfavorable conditions of the water there was some evidence of lowered vitality, in the large numbers of cultures which carried the hydrogen ion concentration[2] to 5, 5.2 or 5.4. What may possibly be another evidence of attenuation is seen in the number of *B. coli* cultures which fail to form indol among those isolated after prolonged exposure to the water.

Many water bacteriologists consider weak gas formation an indication of "attenuation" and therefore evidence of a remote contamination.

This view is supported by some observations on six cultures of the *B. aerogenes* type isolated from this bottle of water. Three of these cultures were isolated when the sample was 47 days old and three at 278 days. All of these cultures gave a normal fermentation in glucose. The three cultures isolated at 47 days produced from 10 cc. of lactose broth approximately 2.7 cc. of gas consisting of carbon dioxide and hydrogen in the ratio of 1.53, 1.54 and 1.48 respectively. This is the normal fermentation for the *B. aerogenes* type. The three cultures isolated at 278 days gave, under similar conditions, 3.77, 3.78 and 3.77 cc. of gas with a ratio of 0.59, 0.56 and 0.51 respectively. These cultures were of course, not identical with those isolated at the earlier date and the evidence of "attenuation" is thus purely circumstantial.

However all of these observations considered together point to a slow change, which may be described as a loss of function, in colon cultures held for a long time in water. This change was perceptible only after the culture had been in water for many weeks.

These results may seem at variance with conclusion reached by Browne who studied changes in bottled water under similar conditions. The conflict is probably only apparent.

Browne's sample was held only 73 days and in that period there was little evidence of change in our sample. Moreover

[2] It should be remembered that the numbers on Sorensen's scale run *inversely as the hydrogen ion concentration.*

Browne used the MacConkey-Jackson system of classification which is merely the possible arrangements of plus and minus signs under sucrose and dulcite and has no relation to the varieties arranged by nature.

CULTURES HELD IN WATER IN PERMEABLE SACS

Conditions more nearly approximating those found when sewage is emptied into streams were obtained by holding cultures or fecal matter in parchment sacs suspended in running water. This was repeated several times both in the laboratory and in small streams, but nearly every experiment came to an untimely end through overheating, freshets or other causes before very complete results were obtained.

TABLE 2

Changes in colon bacteria in running water

AGE	COLON GROUP PER CUBIC CENTIMETER	RATIO B. AEROGENES TO B. COLI
days		
0	190,000	1:2.3
1	130,000	1:4
2	19,000	1:3.3
3	9,000	1:1.2
4	20	1:1.1
7	30	1:0.11

In all cases in which the temperature of the water was relatively high there was an increase in the colon bacteria of both types.

Table 2 shows the results obtained by holding a small amount of dilute sewage in a parchment sac in running water. The sac was made by folding parchment paper around a bottle from which the bottom had been removed. This bottle properly protected, was held in running tap water at a temperature of 16 to 20.6° C.

There was no increase in numbers observed but otherwise these results agreed with those obtained on other sacs under similar conditions. The total number decreased far more rapidly than was the case in the bottle held in the incubator at 20° and

the change in the ratio of *B. aerogenes* to *B. coli* was correspondingly abrupt. The initial determinations showed three or four times as many *B. coli* as *B. aerogenes* but at 7 days there were ten times as many *B. aerogenes* as *B. coli*.

There is a possibility that on account of imperfections in the parchment there was a mechanical loss of bacteria. The results obtained were however consistent and in accord with those reported by other investigators. Moreover it is very improbable that a mechanical loss would have resulted in the relative changes observed in the abundance of the two species.

RELATIVE CHANGES IN COLON-AEROGENES BACTERIA IN POLLUTED STREAMS

It is difficult to even approximate the total number of colon-aerogenes bacteria in a polluted stream but the relative number of *B. coli* and *B. aerogenes* may be obtained with a fair degree of correctness by isolating a considerable number of colonies and determining the group to which they belong by the methyl red test.

This was done on two representative streams. The samples, which were collected by following down the stream in an automobile, were held in ice water and taken to the laboratory at once. They were plated on asparagin agar, a medium on which colon types grow well, but which is not favorable to many other bacteria particularly the streptococci.

One of these streams was Wolf Creek, a rather sluggish stream originating in swamps and flowing through Grove City, a town of about 4000 inhabitants. On its entire course through the town it is rendered stagnant by a dam and is polluted by houses and stables on its banks. At the lower limits of the town it receives the untreated city sewage. Below Grove City it flows through a partly wooded farming country and for 15 miles receives no sewage. Samples were collected at approximately 2 mile intervals and a number of colon cultures obtained from each by direct plating. There is of course an element of chance in picking cultures in this way but the results shown in table 3 are probably fairly representative.

Above the city where there was no sewage pollution all of the cultures isolated were *B. aerogenes*. After flowing through the town, by which it was badly polluted, there was a preponderance of cultures of the *B. coli* type. Two miles below the city sewer there was only 1 *B. aerogenes* culture in 11 isolated. This ratio changed rapidly however, and *B. aerogenes* soon outnumbered *B. coli* though the latter type was still present ten miles below the sewer.

Rock Creek, the second stream investigated, runs into the Potomac river between Washington and Georgetown. It is not so well adapted to this study as Wolf Creek because it receives sewage at various points and the self purification cannot be so

TABLE 3

Relative numbers of B. aerogenes and B. coli in Wolf Creek

SAMPLE NO.	MILES	POLLUTION	CULTURES ISOLATED	RATIO B. AEROGENES TO B. COLI
1	0		7	All aerogenes
2	1.1	Private sewers	15	1.1:1
3	1.1	City sewer	18	0.8:1
4	3.1		11	0.1:1
5	5.1		20	9:1
6	7.1		10	All aerogenes
7	9.1		9	3.5:1
8	11.3		5	4:1

satisfactorily observed. In its upper course it flows through an agricultural country and receives no direct sewage. A few miles above the District of Columbia line the untreated sewage of the village of Kensington is emptied into the stream. There are probably some private sewers before it enters Rock Creek Park in which it is protected from contamination with the exception of two small tributaries, Broad Branch and Piney Branch, both of which are evidently polluted.

The results of the study of Rock Creek are given in table 4. At a point about 10 miles above the district line a sample was taken from which 10 cultures were isolated. All of these were *B. aerogenes*. Two and one-half miles below where the stream passes the small village of Garrett Park nearly one-half of the

cultures isolated were of the *B. coli* type. From a sample taken immediately above the mouth of the Kensington sewer, 3 *B. coli* and 13 *B. aerogenes* cultures, only 2 of which fermented adonite were isolated. A few yards below over half of the cultures isolated were of the *B. coli* type. Of the 13 *B. aerogenes* cultures isolated, 7 fermented adonite which we may assume indicated fecal origin. The fermentation tubes made in the usual way and incubated at 37° did not show a greater number of lactose fermenters immediately below the Kensington sewer than just

TABLE 4

Relative numbers of B. aerogenes and B. coli in Rock Creek

SAMPLE NO.	MILES	POLLUTION	HIGHEST DILUTION SHOWING GAS		CULTURES ISOLATED	RATIO OF B. AEROGENES TO B. COLI	FERMENTATION OF ADONITE BY B. AEROGENES CULTURES	
			24 h.	48 h.			+	−
			cc.	cc.				
1	0		1.0	0.1	10	All aerogenes		
2	2.5	Village without sewers	0.1	0.1	13	1.1 : 1.0		
3	7		0.01	0.01	16	4.3 : 1.0	2	11
4	7	Kensington sewer	0.01	0.01	28	0.86: 1.0	7	6
5	9		0.1	0.01	22	1.0 : 1.0	4	7
6	10.7		0.01	0.01	22	1.0 : 1.0	5	5
7	12.7	Broad Branch	0.1	0.1	17	1.4 : 1.0		
8	14.5	Piney Branch Zoological Park	0.1	0.1	14	1.0 : 1.0		
9	15.7		0.001	0.001	24	1.0 : 1.1		

above it; that is there was gas in 0.01 cc. dilution but none in the 0.001 cc. dilution in each case. Accurate counts could not be made from the plates but they indicated a great increase at this point.

Samples taken at lower points on Rock Creek showed a slight increase in the proportion of *B. aerogenes* but below the mouth of Broad Branch and Piney Branch bacteriological conditions as shown by these tests were nearly as bad as just below the Kensington sewer. This is probably due to a badly polluted condition in Piney Branch.

THE EXAMINATION OF INDIVIDUAL SAMPLES OF SURFACE WATER

In the course of this work about 30 samples of water from a great variety of sources have been examined. These have included samples from grossly polluted streams such as Rock Creek and the Anacostia river and from springs in the Maine woods in which the chance of pollution was remote. With a few exceptions bacteria of the colon-aerogenes type were isolated from these samples without difficulty. The exceptions were a Maine lake without pollution except from a few camps on the shores, a small stream flowing into this lake and which at no point was near a habitation or a highway, a spring flowing out

TABLE 5

Comparison of cultures from grain, water and feces

SOURCE OF CULTURES	TOTAL CULTURES	B. COLI TYPE	B. AEROGENES TYPE	B. CLOACAE TYPE	B. AEROGENES TYPE				B. CLOACAE TYPE				AVERAGE GELATIN LIQUEFICATION
					Indol	Mannite	Dulcite	Adonite	Indol	Mannite	Dulcite	Adonite	
					per cent +	per cent +	per cent +	per cent +	per cent +	per cent +	per cent +	per cent +	mm.
Grain....	159	8	111	40	7.20	20.72	16.21	12.61	0	97.5	100	0	5
Water....	134	54	67	13	30.98	91.04	23.88	71.21	15.38	84.62	15.38	58.33	16
Feces.....	177	131	46	0	21.74	100.0	21.74	100.0					

of the gravel on the shore of the lake and a well protected spring in Rock Creek Park, Washington. In all other cases at least 2 or 3 colon cultures were obtained by direct plating.

A total of 134 cultures were isolated from these samples. In table 5 the characters of these cultures as a group are compared with those from grains and from human feces. There will be noticed a general tendency for the water cultures to agree with those of fecal origin rather than with those isolated from grains.

In making this comparison it should be remembered that the grain cultures included some which were very probably of fecal origin while the water cultures included some evidently not of fecal origin. The 12 per cent of the *B. aerogenes* cultures from

grains which fermented adonite had all the characters of the *B. aerogenes* cultures from feces. If these tabulations should be made on this basis, it would be found that the cultures of the fecal *B. aerogenes* type from water would agree very closely with those from feces while they would be quite distinct from the grain cultures. There is a decided difference in the characteristics of the liquefying cultures from grains and from feces but in the light of our present knowledge of this sub-group, it would be unsafe to make any definite deductions from these data. Greenfield, (1916) found that of 405 cultures from ground and surface waters 70 per cent were of the *B. coli* type as indicated by the methyl red and Voges-Proskauer tests.

More light can be thrown on the value of a qualitative examination on the colon bacteria by a study of the results from individual samples. Space will not permit a consideration of all the samples but a few representative ones are given.

No. 21. Rock Creek. This is a polluted stream previously described. Two samples were taken from which 13 cultures were isolated. Eleven of these were high ratio, gelatin −, indol −, adonite +, dulcite −, sucrose and salicin +. One differed from these in being indol + and dulcite +. The characters of these cultures agreed very closely with those of the high ratio cultures isolated from human feces.

In view of the results obtained in the survey of Rock Creek it is rather surprising that no *B. coli* cultures were obtained from these samples. The samples from which *B. coli* were isolated were taken about a year later than those giving all *B. aerogenes*. The disappearance of the *B. coli* type may be looked upon as evidence of self purification and in this the time element is an important factor. The rate of flow which varies greatly in a small stream has a direct influence on the time for which sewage is exposed to purifying influences before it reaches a given point. There is also a possibility that the pollution in the lower part of Rock Creek may have become materially increased after the first samples were taken.

No. 10. The Potomac River. The pollution of this river has been very thoroughly studied (Cumming, 1915). The principal source of pollution is the city of Cumberland about 180 miles above Washington. Sewage is emptied into the river or its tributaries at other points nearer Washington but, considering the volume of water flowing in the river they are relatively unimportant.

Ten cultures were obtained from two samples collected near Washington when the river was in normal flow. These included three cultures of the *B. coli* type which were typical in every way except that they had a hydrogen ion limit of 5.2 to 5.4. In this regard they corresponded to the cultures held in water many weeks rather than with freshly isolated fecal cultures.

The 7 *B. aerogenes* cultures included 5 which fermented adonite and starch and otherwise agreed with the fecal type.

No. 30. Anacostia River. One sample was taken at the bridge below Bladensburg when the flow was above normal. This stream is polluted by the sewage of Hyattsville and other smaller villages. All of the nine cultures were of the *B. coli* type and had a hydrogen ion limit of 4.8 to 5.3.

These results indicate a high pollution of recent origin, comparing as they do with results obtained a short distance below the sewer in Rock Creek and Wolf Creek.

No. 28. Pimmit Run. This is a relatively small stream flowing into the Potomac at Chain Bridge. It probably receives no direct sewage but flows through an agricultural country from which it is contaminated by surface wash. No *B. coli* cultures were obtained from the one sample examined but of the 8 *B. aerogenes* cultures isolated 6 fermented adonite and starch and were probably of fecal origin. One fermented starch but not adonite and one fermented neither adonite nor starch.

No. 26. Spring near Chain Bridge. This spring is in a rather sparsely settled suburban district and was carefully protected from surface contamination by tiles and stone work. The source of the water was not evident and the possibility of contamination was a matter of conjecture.

The houses in the vicinity were, for the most part connected with sewers. Five *B. aerogenes* cultures were isolated, all of which fermented adonite and starch and were therefore of the fecal type.

No. 11. Spring near Little Falls. This spring at a camp on the Virginia shore of the Potomac was carefully protected by stone work. The shore is wooded for nearly a half mile from the river. The camps in the vicinity are occupied at irregular intervals and there are no houses within a mile.

The spring flows from gravel at the foot of an abrupt rocky hill and so far as an examination of the surroundings shows there is no reason to expect contamination. Of the 5 cultures isolated 3 were *B. coli* and two were *B. aerogenes* fermenting adonite and starch.

No. 6. Davis Brook. This is a very small stream in the Maine woods. About 1 mile above the point where the sample was taken it flows from a small but very deep pond fed by submerged springs. On the upper end of this pond is a summer camp. Considering the very small flow from the pond there is only a remote probability of any contamination from this camp affecting the stream.

One of the 4 cultures isolated was of the *B. coli* type. Two of the *B. aerogenes* cultures did not ferment adonite or starch and one fermented starch but the fermentation of adonite was not determined.

Lactose broth tubes inoculated with 1 cc. of water gave small amounts of gas in forty-eight hours.

No. 5. Small Brook. This is a very small stream flowing through dense woods except that at about a mile above where the sample was taken it crosses a highway.

Lactose broth inoculated with 10 cc. of water gave a small amount of gas in forty-eight hours. Of the 4 gas forming cultures isolated 2 were of the *B. coli* type, one was a high ratio liquefier and one was a *B. aerogenes* which failed to ferment adonite.

CONCLUSIONS

Through its greater resistance to the unfavorable conditions found in water the *B. aerogenes* type is able to survive longer than *B. coli*. This was apparent in the water held in bottles, in the sewage held in parchment sacs, in running water and in polluted streams. From this we may draw the inference that water near the source of pollution should contain a greater proportion of *B. coli* to *B. aerogenes* than after the processes of self purification have had an opportunity to act. This was found to be the case in two sewage polluted streams. In each case the gas forming bacteria isolated above the source of pollution consisted largely of *B. aerogenes* cultures, while immediately below the sewers a majority of the cultures isolated were of the *B. coli* type.

At lower points on the streams the proportion of *B. aerogenes* increased again. In the only case in which suitable determinations were made it was found that a similar relation existed between the fecal and nonfecal types of *B. aerogenes*.

The assumption that the two types, *B. coli* and the fecal *B. aerogenes*, are distinctively fecal organisms without other habitat may make it difficult to explain their occurrence in certain samples of water in which the chances of contamination seem very remote. We have isolated both the *B. coli* type and the fecal *B. aerogenes* type from water in which the chances of pollution from dwellings or wash from farm lands is almost completely excluded. In some cases the fact that all of the cultures isolated belonged to one or the other of the two fecal types would point to a source of contamination not found by physical examination of the surroundings.

In no case was the possibility of contamination by animals completely excluded. This is especially true of the springs and brooks in the Maine woods. Deer and moose frequent water courses in the warm months and there are a number of kinds of small animals which make their homes along the banks.

Even a protected spring may be exposed to the visits of squirrels and similar animals. It is possible that the occasional colon bacillus of the fecal type found in waters presumably free from pollution may be accounted for in this way.

The possibility of fish as a source of intestinal bacteria in water is suggested by the work of Browne, (Browne 1917), who found *B. coli* in the intestinal tract of 39.8 per cent of scup examined. The feeding habits of the fish may determine the presence or absence of colon-aerogenes bacteria in its digestive tract but in no case is it likely that fish would account for more than occasional cultures.

There is also a possibility that the digestive tract of animals is not the only source of the so called fecal type of colon. At the present time there is little or no evidence that this is the case. It is true that some of our water cultures were not true to the fecal type and therefore might suggest a different variety or source. These differences were very slight, consisting for the most part in failure to form indol or in a hydrogen ion limit slightly lower than that of the typical culture.

Whatever the final conclusion may be in regard to the occurrence of these occasional cultures the fact remains that there

are two types of the colon-aerogenes group which occur in fecal matter in large numbers. While it is possible that they may also live in the soil or other material from which they may be carried to water, their presence in water is strong presumptive evidence that the water was polluted with fecal matter. One of these types has certain distinctive characters which render identification easy; the other type is not so well marked but may be identified with reasonable certainty and without great difficulty.

In one way the recent contributions to the knowledge of the colon-aerogenes group has not changed the methods of water bacteriology. The presence of any particular kind of bacteria in water is merely an indication of the existence of certain conditions and the bacteriologist must weigh all the available evidence on the basis of his experience and make his decision accordingly. However, the method which we now have of separating the colon-aerogenes group into varieties which have a very definite relation to habitat should be of material assistance in forming an opinion of the potability of a water. The value of this ability to separate the varieties of the colon-aerogenes group is much more evident if a sufficient number of cultures can be isolated from each sample to establish the relative numbers of the different types. This, we believe will prove to be of much greater value than the mere determination of the presence of colon or of any one variety of colon.

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THE ELIMINATION OF SPURIOUS PRESUMPTIVE TESTS FOR B. COLI IN WATER BY THE USE OF GENTIAN VIOLET

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The bacteriological criterion of potable water is the *absence* of *B. coli* and its close allies,—aerobic, Gram-negative, non-sporulating bacilli or coccobacilli which produce acid and usually gas in media containing lactose, and which do not liquefy gelatin.[1] The best test for purity of water, according to standard methods (1917) is a negative presumptive test, i.e., failure of gas formation in lactose broth; water, which in quantities of 10 cc. or more, does not produce gas, may be unreservedly accepted for domestic use. Even if 1 cc. of a given supply yields a negative result, though colon bacilli are present in larger quantities, the water should probably be regarded as merely suspicious. Only the extreme difficulty of ascertaining the presence of the various intestinal pathogens makes us rely upon the more actively fermenting colon bacillus as a criterion of potential pollution.

While a negative result permits very definite statements as to the purity of the sample, a positive result, i.e., the formation of gas in the presumptive test, does not necessarily condemn the water. Even if the gas produced be proven due to *B. coli* the water may be quite safe if no pathogenic organisms have entered the water along with the colon bacillus. Thus the colon bacilli may have had their origin in perfectly healthy human beings free from disease producing micro-organisms, in

[1] In this paper the term *B. coli* is used to include all members of the colon groups as above defined—*B. acidi-lactici*, *B. aerogenes*, *B. communior*, *B. communis*, and their subvarieties.

birds or lower mammals whose intestinal diseases are not ordinarily transferable to man, or even in certain non animal sources, e.g., grains. Methods have recently been described which promise some degree of differentiation of certain of these forms; the literature was fully reviewed by Winslow (1916) and need not be repeated in detail here. While fully appreciative of the refinements in interpretation which the work of Rogers, Clark and Davis (1914), Rogers, Clark and Evans (1915), Clark and Lubs (1915), and others makes possible, we are convinced that an equally important problem concerns the use to be made of the presumptive test. We are not willing to discard this test as Professor Winslow advises in case of water of fair quality; indeed we feel that inasmuch as negative presumptive tests are most significant it is in just this type of water, even more than in badly polluted water, that the presumptive test is of most value. We fully agree however as to the need of the colon confirmatory test where the presumptive test is positive. Finally, and most important, the finding of *B. coli* in water should properly be interpreted *only* as an indication of the absolute necessity of a sanitary survey to locate, identify, and, if possible, eradicate the source of pollution.

In a varying proportion of cases, subcultures from positive presumptive tests upon the litmus lactose agar plate for the purpose of isolating and identifying the gas former fail to yield acid forming colonies. In these instances it is customary to interpret the result as non-significant so far as concerns pollution, since Creel (1914) has pointed out the rôle of sporulating lactose fermenting anaerobes in the presumptive test, a point which the latest standard methods seem not to have emphasized sufficiently. Even when Whipple (1903) used the glucose broth presumptive test following its original suggestion by Theobald Smith (1893) (1895) and Jackson (1906, 1907), modified this by substituting lactose bile, it was known, according to Winslow (1916), that certain organisms other than *B. coli* might be responsible for positive tests, but the factor of error introduced thereby was believed to be nearly negligible. Unfortunately Whipple's paper, which we quote from Professor Winslow's, is not available, but

it is clear that Jackson did not appreciate the importance of the anaerobes when he said, "no gas producer or mixture of gas producing bacteria will give results as high as 25 per cent gas except *B. coli*, even when three days' incubation is employed." Further experiences have shown the necessity of not relying simply upon the presumptive test as a criterion of pollution; Fuller (1915) found a proportion of positive presumptive tests in which *B. coli* could be confirmed ranging from only 60 to 90 per cent in 1 cc. samples, and Cumming (1916) found a percentage of *B. coli* to total gas formers ranging from 47.5 to 96.3. Moreover the amount of gas produced is no criterion, as is suggested, even in the most recent Standard Methods, since many of the sporulating anaerobes completely fill the closed arm of the fermentation tube with gas.

A recent experience by the Bureau of Sanitary Engineering of the State Board of Health of California (1916) at Sacramento is pertinent, where doses of chlorine so excessive as to cause disagreeable tastes and odors were being used by the local authorities to destroy *all* lactose fermenting bacteria in the water in the belief that this was necessary to insure its freedom from typhoid bacilli. It was indeed shown that the dosage of chlorine could be safely reduced from 2.6 pounds per 1,000,000 gallons to 1.3 pounds in fairly clear water (30 parts per million turbidity—for the dosage has to be varied according to turbidity), eliminating *B. coli* and presumably the typhoid bacillus, if present, without leaving any trace of free chlorine. With water so treated, however, presumptive tests were frequently positive, due to certain sporulating anaerobes from among which Mr. Frank Bachman succeeded in isolating *B. welchii*, a culture of which he has kindly given us.

SELECTIVE INHIBITION IN THE PRESUMPTIVE TEST

It is the purpose of this paper to describe a method to prevent gas formation by these anaerobic organisms, so that a greater proportion of positive tests is referable to coliform bacilli alone. This we have proposed to accomplish through

selective inhibition by gentian violet. Incidentally the Gram-positive sporulating aerobes, among which occasional forms capable of giving positive presumptive tests occur, are also inhibited.

The early literature dealing with the germicidal and antiseptic action of dyes has been sufficiently reviewed in a former publication (1914). Churchman's (1912) work, which was the point of departure for that paper, and which enabled him to state that a majority of Gram-positive organisms are inhibited in their growth by gentian violet, whereas most Gram-negative organisms grow well in its presence, dealt mostly with aerobic cultures. Certain anaerobes, e.g., two strains of *B. tetani*, were found by him to follow the rule as we have already amply confirmed (Hall and Taber, 1914). But others, namely *B. welchii* and *B. sporogenes*, were noted as exceptions to the general rule, i.e., while Gram positive, they were classed as resistant to the dye in the concentration used.

Inasmuch as we have undertaken a comparative study of a series of cultures of organisms belonging to the group of Gram-positive sporulating anaerobes, it seemed that it would be of interest to determine what, if any, difference could be found in their behavior and growth in the presence of gentian violet. As often happens, this study, which was undertaken purely from academic motives, was nearly completed before we appreciated its very pertinent practical utility.

THE ACTION OF GENTIAN VIOLET UPON CERTAIN GRAM POSITIVE SPORULATING ANAEROBES

The cultures of anaerobes tested came from various sources as indicated below. Most of them have been tentatively identified but we do not wish to emphasize the question of identity of any, except such as are designated "confirmed," since we have found certain atypical features in some of the cultures which may necessitate re-naming them. All are Gram-positive sporulating obligative anaerobic bacilli and, with the exception of *B. tetani* and *B. putrificus*, of clostridial morphology. The

cultures are free from aerobic contamination, but in some cases we feel certain two or more species of anaerobes are present in a single culture. For the purpose of this paper we have not yet undertaken to purify them—hence our hesitancy to emphasize the question of identity.

The first tests were made in deep agar according to the technic used by one of us (I. C. H.) with Mr. Taber (1914) in connection with the tetanus bacillus, but this proved not to be feasible in studying a larger number of cultures, owing to the care necessary in preparing and inoculating the tubes; it was replaced by the simpler method of tubing the media, after the desired concentration of dye had been secured by addition of the proper amount of a 1 per cent solution followed by sterilization in the Arnold sterilizer. In some of the earlier tests, crude glucose was used by oversight, resulting in decolorization which we attribute to residual SO_2 used as a bleaching agent in the preparation of the glucose and which is known to reduce dyes of the gentian violet group to their leuco-bases. A medium made with chemically pure glucose does not decolorize gentian violet. The reaction of the media is no doubt important, although stronger acids are required to decolorize gentian violet than is the case with alkalis; the reaction was adjusted to +1. Heavy seed cultures were prepared in the constricted tube described by Hall for the cultivation of anaerobes in glucose broth (1915) After twenty-four hours incubation at 37°C., 1 cc. was transferred to the tubes of melted glucose agar with the dye cooled to 40 to 45°C—rolled to mix thoroughly and incubated at 37°C. Control tests were always made in similar media without dye. Control tests for aerobic contamination were also made from each seed tube on agar plates in each experiment. Further, all positive growths were subcultured on agar slants or plates to detect possible aerobic contamination. All of the organisms produce gas even in the absence of fermentable carbohydrates; this fact and the appearance of definite colonies in the depths served as the criteria of growth. In all cases also, growth is accompanied by decolorization of the dye. The results of the tests are tabulated in table 1.

TABLE 1

Action of gentian violet upon certain Gram-positive sporulating anaerobes

CULTURE NUMBER	SOURCE	LABELLED	CONFIRMED	CONTROL NO DYE	GENTIAN VIOLET		
					1-100,000	1-10,000	1-1,000
51	Am. M. Nat. Hist.	B. botulinus no. 595	Yes	+	+	− − − − −	− − − − −
77	Cow dead of blackleg		B. chauvei	+	+	− − − − −	− − − − −
80	(*)	B. tetani	No—non-toxic	+	−+	− − − − −	− − − − −
82	Sterility test			+	−+	− − − − −	− − − − −
83	(*)	B. oedematis-Pasteur	No—probably B. chauvei	+	+	− − − − −	− − − − −
84	Dr. K. F. Meyer	B. chauvei-Munich	Yes	+	+	− − − − −	− − − − −
86	(*)	B. oedematis-Pasteur	Doubtful—contaminated?	+	+	− − − − −	− − − − −
89	Autopsy no. 1535. Gas infection			+	−+	− − − − −	− − − − −
105	(*)	B. tetani	No—non-toxic	+	+	− − − −+	− − − − −
113	Dr. K. F. Meyer	B. oedematis-"Koch"	Yes	+	+	+	− − − − −
114	Dr. K. F. Meyer	Ghon-Sachs	Yes	+	+	− − − − −	− − − − −
115	Dr. K. F. Meyer	Vibrion Septique	Yes	+	+	− − − −+	− − − − −
133	Am. M. Nat. Hist.	B. welchii no. 521	Yes—but contaminated	+	+	+	− − − − −
134	(*)	B. sporogenes 120	Doubtful	+	+	+	− − − − −
136	(*)	B. tetani	No—non-toxic	+	+	− − − − −	− − − − −
139	Rabbit infection			+	− − − − −	− − − − −	− − − − −
141	Rabbit infection			+	+	+	− − − − −
165A	Dr. E. C. Dickson	B. botulinus	Yes	+	+	− − − −+	− − − − −
165B	Dr. E. C. Dickson	B. botulinus	Yes	+	+	− − − − −	− − − − −
165C	Dr. E. C. Dickson	B. botulinus	Yes	+	−+	− − − − −	− − − − −
178A	Canned corn		B. botulinus	+	+	−+	− − − − −

| | | |

None of the cultures grew in glucose agar containing 1–1,000 gentian violet and only 14 out of 33 showed growth in a concentration of 1–10,000. Of these latter 6 grew within one day, 2 within two days, 1 in three days and 5 only within five days. In all of these there was marked evidence of restriction by the dye. In a concentration of 1–100,000 all the cultures but 2 grew—mostly quite vigorously, with gas formation and marked decolorization of the dye. All the controls grew vigorously except 3 cultures of *B. putrificus* which, not fermenting glucose, are characterized by somewhat more sluggish development. The tests were repeated with most of the cultures three times with essentially similar results.

In spite of the fact that according to the above tests more than 1 part gentian violet in 10,000 of agar is required to completely inhibit the growth of these organisms, we conceived that a smaller amount might suffice to inhibit spurious presumptive tests in lactose broth due to organisms of this group. There are two reasons supporting such a supposition, first, the conditions of anaerobiosis in the Durham fermentation tube are not so favorable, second, it is believed that the colloidal nature of the agar reduces the efficiency of the dye, thereby making a higher concentration necessary to accomplish a given result.

A test of these organisms was therefore made in Durham fermentation tubes with 1–100,000 gentian violet in 1 per cent lactose broth. The test was controlled by means of a series of tests without the dye, all of which developed growth excepting cultures 115 and 192; their failure is attributed to the fact that in these tests no special precautions were taken to eliminate oxygen. All but six of the control tests produced gas in addition to turbidity. Of the dye tests however only one produced either gas or turbidity and this was shown to be aerobically contaminated. It may be mentioned that the somewhat inferior anaerobiosis of the Durham tube for pure culture of anaerobes is compensated in routine presumptive tests for *B. coli* in water by the practically constant presence of aerophilic organisms which reduce the oxygen tension sufficiently to provide suitable conditions of anaerobiosis. We may admit also that the writer's

modification (1914) of the Durham fermentation tube is less apt to provide anaerobic conditions than the usual type.

In order to test the applicability of gentian violet in actual water examinations, arrangement was made to secure samples showing positive presumptive tests from the Bureau of Sanitary Engineering of the State Board of Health of California. Our sincere thanks are due Messrs. C. G. Gillespie and Frank Bachman for their courtesy in this matter. Inasmuch as the samples were frequently several days old before we received them it is not surprising that our findings do not coincide exactly.

TECHNIC

Upon receipt the bottles were thoroughly shaken and the water divided into two parts; one portion was heated to 56°–60°C for thirty minutes, the other being left unheated. Each of these was again subdivided into two parts, one of which was inoculated into a Durham fermentation tube containing 2 per cent lactose broth with gentian violet, the other being inoculated into a tube of similar medium without gentian violet. The tubes contained 10 cc. of double strength media and the amount of water inoculated was 10 cc. in each case. The final concentration of lactose was therefore 1 per cent; the final concentration of dye was 1–100,000 in the first 21 samples, after that 1–20,000. Tests at the latter concentration were also made with what remained of the first 21 samples. The change was made because gas was encountered several times in the unheated sample inoculated into dye broth from which no aerobic gas former could be isolated; we interpreted this result as due to imperfect inhibition of gas forming anaerobes, indeed we had anticipated that *B. coli* should be isolated from every unheated sample showing gas in the presence of 1–100,000 gentian violet. As later shown even 1–20,000 is not sufficient to give this result, due, we believe, to the somewhat better conditions of anaerobiosis provided by the presence of aerobic organisms.

Incubation was at 37° until gas formation occurred, if within five days. Plates of litmus lactose agar were then streaked

and acid-forming colonies isolated upon the unused half of the plate. After all but 22 samples had been examined we began to realize that there are possibly two conditions in which the positive presumptive test may truly be due to *B. coli* and yet great difficulty be encountered in the isolation of the proper organisms due to the fact that no red colonies appear on the litmus lactose agar plate.

The first of these is the occurrence of so-called "attenuated" coliform bacilli which may be considered to have temporarily or partially lost their property of fermenting lactose under aerobic conditions. Many water analysts are well aware of this occurrence and so the new standard methods for 1917 emphasize the necessity of testing suspicious looking surface colonies of true morphology in lactose broth for gas formation, even though they do not produce acid upon the litmus lactose agar plate. But it is to a degree only accidental that this procedure sometimes results in identification of *B. coli;* in our experience blue surface colonies usually, but not invariably, fail to form gas in the fermentation tube. Our work holds no suggestion as to the elimination of this difficulty.

The second condition is that in which strongly proteolytic organisms, e.g., certain of the hay bacillus group, not fermenting lactose, liberate sufficient alkali to neutralize any acid due to *B. coli* in the plates, thus suppressing the appearance of red colonies except when widely separated; a further aggravation is that these organisms sometimes have marked spreading proclivities. Fortunately the use of gentian violet is quite efficacious in preventing this particular source of trouble, since most aerobic sporulating bacteria are inhibited as well as the Gram-positive sporulating anaerobes. It was quickly determined that the addition of 1–100,000 gentian violet to litmus lactose agar served admirably to inhibit the undesirable organisms, not always completely, but at any rate sufficiently to prevent their spreading and interfering with the display of acid by *B. coli.* The color imparted by this amount of dye is quite insufficient to mask the desired color changes in the litmus. In advocating the use of gentian violet in this manner for the

isolation of *B. coli* we appreciate of course that the method is essentially the same as that used by Drigalski and Conradi (1902) in the isolation of *B. typhosus*, only the aim is slightly different.

Isolated coliform colonies were stained by Gram's method as modified by Stovall and Nichols (1916) and if typical Gram negative coccobacilli were present, they were tested in lactose broth for gas formation and in gelatin for non-liquefaction. These confirmatory tests were made at 37° C. for forty-eight hours, gelatin being tested for solidification by immersion in ice-water. In case only non-acidifying colonies appeared, at least two were similarly isolated and if otherwise coliform were tested in lactose broth to detect so-called "attenuated" *B. coli*, as required by the 1917 Standard Methods for the Examination of Water and Sewage.

Since our hypothesis involved the demonstration of *B. coli* in as many cases as possible where gas was produced in the presence of gentian violet, we adopted a uniform procedure of repeating the above tests with a subculture into a new tube of lactose broth from the original presumptive, if the first plate failed to show typical coliform acidifiers, or if the identification of *B. coli* failed, in either of the tests of the unheated sample.

The tabulation of the complete data occupies so much space that it seems best only to summarize the findings for the sake of economy, as in table 2.

Unquestionably the most interesting feature in table 2 is the behavior of the heated samples. In only one case was a positive presumptive test secured in the broth containing the dye as against 18 positive tests without it; in fact practically all of the tubes with dye appeared to be sterile. Aerobic spores as well as anaerobic spores were inhibited. The single tube mentioned showed slight gas only after four days' inoculation and this was found to be due, neither to *B. coli* nor necessarily to anaerobes; a lactolytic acid and gas forming sporulating bacillus was secured from the plates. This culture in fact was the starting point of our use of gentian violet in the lactose agar plate inasmuch as repeated tests from the broth failed to show

the slightest growth upon such a plate. The same organism was isolated from the presumptive test without gentian violet, and we believe its presence in the unheated sample was the reason for our failure to secure *B. coli* from this sample, from the presumptive test either with or without gentian violet although on the occasion of two other independent tests *B. coli* was isolated from the same sample. At least 16, or 76 per cent, of the 21 samples contained gas-forming anaerobes, as shown by positive presumptive tests in plain lactose broth with the heated samples from which only non-lactolytic aerobes appeared on the litmus-lactose-agar plate subcultures; these were stained by

TABLE 2

Tests for B. coli and sporulating gas-forming anaerobes in 21 samples of water—using 1–100000 gentian violet in the presumptive test

TREATMENT OF SAMPLE	SAMPLES SHOWING GAS IN LACTOSE BROTH	SAMPLES SHOWING ACID COLONIES ON LITMUS LACTOSE AGAR	SAMPLES FROM WHICH B. COLI WAS ISOLATED AND IDENTIFIED		
			First trial	Second trial	Total
Not heated	1–100,000 gentian violet, 20	14	11	4	15
	No gentian violet, 21	12	8	4	12
Heated 56°–60°C.; 30 minutes	1–100,000 gentian violet, 1	1*	0	0	0
	No gentian violet, 18	2*	0	0	0

* Gas in presumptive tests and acid on plates due to lactolytic aerobic spore bearers.

Gram's method and were found to be Gram-positive sporulating bacilli with one exception which contained Gram-positive cocci. Two heated samples also giving positive presumptive tests in plain lactose broth yielded acid colonies on litmus lactose agar; the organisms were lactolytic aerobic spores. Anaerobic spores may also have been present but we cannot be sure, and the percentage of samples certainly having anaerobic spores is given exclusive of these two.

As table 2 shows, all the samples showed gas in the plain lactose broth tube inoculated with 10 cc. unheated water, as against only 20 in the gentian violet lactose broth. But of the 20 there were 14 which produced acid colonies on litmus-lactose-

agar, as against 12 out of the 21. And of the plates from the gentian violet presumptive tests, 15 samples yielded *B. coli* whereas only 12 of the samples tested on plain lactose broth yielded *B. coli*. The discrepancy in favor of gentian violet in the presumptive test is still further shown in the greater proportion of cultures secured in the first test, 73 per cent from the dye series, as against 67 per cent from the non-dye presumptive. Strangely enough, the odd sample failing to show a positive presumptive test in the presence of gentian violet yielded *B. coli* from the non-dye tube. Looking ahead to the data formulated in table 3 in which gentian violet 1–20,000 was used we find that this particular sample yielded *B. coli* from the test, with, as well

TABLE 3

Tests for B. coli and sporulating gas-forming anaerobes in 20 samples of water—using 1–20,000 gentian violet in the presumptive test

TREATMENT OF SAMPLE	SAMPLES SHOWING GAS IN LACTOSE BROTH	SAMPLES SHOWING ACID COLONIES ON LITMUS LACTOSE AGAR	SAMPLES FROM WHICH B. COLI WAS ISOLATED AND IDENTIFIED		
			First trial	Second trial	Total
Unheated	1–20,000 gentian violet, 15	5	4	8	12
	No gentian violet, 20	7	5	9	13
Heated 56°–60°C.; 30 minutes.....	1–20,000 gentian violet, 0	0	0	0	0
	No gentian violet, 19	1	0	1	1

as without the dye. We cannot explain this anomaly. Our failure to isolate *B. coli* from five unheated samples showing gas in the presumptive test led us to increase the concentration of dye to 1–20,000. The unused residues of 20 of the first 21 samples were therefore re-examined with this change in technic along with new samples secured. Table 3 summarizes the results of this second series of tests with these 20 samples. Part of the differences in findings are no doubt due to the greater age of the samples when the last tests were made.

This time as in the first instance the selective inhibition of sporulating gas formers is shown in the case of the heated sample tested in lactose broth containing gentian violet; not one showed

any trace of gas in five days. Inasmuch as only one of the 19 heated samples showing gas in the test without the dye produced acid on the lactose agar plate, due incidentally to *B. coli* which was isolated and must be considered to have escaped the heating, we are justified in the statement that at least 90 per cent of these samples contained gas forming sporebearing anaerobes, and considering the two series of tests, we can say that only one of the whole 20 samples failed both times to show gas necessarily interpreted as due to anaerobic sporebearing bacilli; in other words 95 per cent of these particular samples contained organisms in addition to *B. coli*, capable of giving rise to a positive presumptive test in lactose broth without gentian violet. The organisms growing upon the litmus lactose agar plates, though non-acidifying, barring the one sample in which *B. coli* was found, were stained by Gram's method and were found in every case to be Gram-positive sporulating bacilli of the hay bacillus group.

All the unheated samples showed gas in the standard presumptive test as against 15 in the gentian violet lactose broth. The proportion showing acid colonies from each set was approximately one-third—considerably lower than in the first series, owing to the disappearance of *B. coli* from some of the samples on standing. Notwithstanding the low proportion of samples showing acid colonies, we were able to isolate *B. coli* from 13 of the 15 showing gas in dye broth and from 14 of the 20 showing gas in plain lactose broth. One sample showing *B. coli* in the standard test failed to show it in the dye test. From the other sample showing gas in gentian violet lactose broth a culture tentatively designated as *B. cloacae* was isolated. This organism, as usually described, differs from *B. coli* only in possessing the ability to liquefy gelatin. We have had a number of such cultures which will be mentioned in the further discussion. In each case we have attempted, and usually successfully, to separate such cultures into subcultures, one of which liquefies gelatin and does not ferment lactose, the other of which ferments lactose and does not liquefy gelatin; in other words, *B. coli*. The principal organisms thus separated from *B. coli* have

been identified as *B. proteus* or *B. fluorescens-liquefaciens*. In all cases the original colonies were well separated on the plate, and usually we have been at a loss to account for the association of two separable species in a supposedly pure culture giving the reactions ascribed to *B. cloacae*. In some other cases contaminations subsequent to the primary isolation were responsible; this was assumed in those few instances when the gelatin liquefier proved to be a spore bearer. At any rate we have come to question whether so-called *B. cloacae* is not often a mixture of a gelatin liquefying non-lactose fermenter with *B. coli*. In our studies all such organisms were planted on plain agar and set aside for further study. Unfortunately in some of them we lost one of the organisms, or must we say, the culture lost its lactolytic *properties?* In the case first referred to above the culture would not ferment lactose when re-tested, though it had formerly done so actively; it conformed therefore to the usual description of *B. proteus* and we conceive that we lost *B. coli*. In one other case of this series we clearly separated *B. proteus* from *B. coli* by plating out in gelatin from a fermentation tube of lactose broth inoculated with what appeared to be *B. cloacae*.

An interesting point in this second series is that the proportion between 1st and 2nd trials at isolation of *B. coli* is reversed, as compared to the 1st series, over one-half of the successful isolations coming from the second trial, indicating, we believe, a loss in number and vigor of *B. coli* due to storage. There were three samples in which *B. coli* was found the first time but not the second; the smallest amounts of water in which *B. coli* could originally be found, according to the Bureau of Sanitary Engineering were respectively 10 cc., 1 cc., and 0.1 cc. There were two samples originally containing *B. coli* in 10 cc. and 1 cc. respectively in which *B. coli* was found the second time and not the first and one sample originally showing *B. coli* in 10 cc. in which we never found it. As against these data showing the disappearance of *B. coli* from standing samples of polluted water we have only one case in which the presence of gas forming anaerobes could not be corroborated in the repeated test.

Since 5 samples, unheated and tested in gentian violet lactose broth failed to produce gas in five days, we conclude that *B. coli*, present as shown by previous tests, had disappeared from the sample. All 5 showed gas in the presumptive test without gentian violet—interpreted in all but one, from which *B. coli* was isolated with some difficulty—as due to anaerobes.

In addition to the last tests, examinations were made of 22 fresh samples, according to the same technic. The data of table 4 are comparable, therefore, to those of table 3 except that the samples had not stood so long.

TABLE 4

Tests for B. coli and sporulating gas-forming anaerobes in 22 samples of water—using 1–20,000 gentian violet in the presumptive test

TREATMENT OF SAMPLE	SAMPLES SHOWING GAS IN LACTOSE BROTH	SAMPLES SHOWING ACID COLONIES ON LITMUS LACTOSE AGAR	SAMPLES FROM WHICH B. COLI WAS ISOLATED AND IDENTIFIED		
			First trial	Second trial	Total
Unheated	1–20,000 gentian violet, 18	14	14	1*	15
	No gentian violet, 22	12	12	1*	13
Heated 56°–60°C.; 30 minutes	1–20,000 gentian violet, 0	0	0	0	0
	No gentian violet, 22	1†	1†	0	1

* Same sample—"attenuated" B. coli fermenting lactose anaerobically but not aerobically.

† Same sample—B. coli not killed by heating.

Analysis of the data shows that this series of samples possessed anaerobic sporulating gas formers in at least 19, or 95 per cent, of the samples according to the standard presumptive test in lactose broth with the heated specimens. In the odd case *B. coli* which had escaped the heating was isolated; its occurrence in the standard test, and not in the dye test of the heated sample, must have been fortuitous since the organisms could be shown not to be inhibited by gentian violet. There were no positive presumptive tests with the heated sample having gentian violet. Of the positives in the standard test 12 were tested on a lactose agar plate containing 1–10,000 gentian violet; in only one case did growth appear, which consisted of a mixture of thick Gram

positive and thin Gram negative sporulating rods. An attempt at repeated demonstration of the non resistance of this particular culture to gentian violet failed, but this and other observations show that exceptional strains of hay bacillus may be found which are not inhibited completely by gentian violet.

In the unheated samples there were 11 in which *B. coli* was isolated from both the dye and the non-dye tests. In the case of one of these in gentian violet lactose broth 2 cultures, apparently *B. cloacae*, were isolated from well separated acid colonies on litmus lactose agar. One of these was subsequently separated into 2 subcultures having the properties of *B. fluorescens-liquefaciens* and *B. coli* respectively; the other after a delayed period was found to have lost the property of *B. coli*, i.e., lactose fermentation, formerly possessed, and retained only the characteristics of *B. fluorescens-liquefaciens*. Our interpretation of this phenomenon has already been discussed.

Four unheated samples yielded *B. coli* from the dye test and not from the standard test. In 2 of these the plates were clearly overgrown by spores which we feel so masked the colonies and acid produced by *B. coli* that we could not pick out the colonies; in the other 2 no acid was displayed in either the first or second tests, and the picked colonies proved not to be the colon bacillus.

Two unheated samples yielded *B. coli* from the standard test and not from the dye test. In one of them the presumptive test was positive only after three days, and the non-acid colonies picked from the plate failed to ferment lactose; the second presumptive test was negative for five days. In the other case a culture tentatively called *B. cloacae* was isolated; subsequently examined with a view to the separation of the unheated *B. coli* from the gelain liquefier, it was found that the culture presented only the characteristics of *B. proteus*.

Five unheated samples failed to yield *B. coli* from either the standard or the dye test. In 4 of these the presumptive test with dye was negative during five day's incubation; the fifth showed a bare trace of gas on the fifth day only. In the standard test all showed gas, 1 on the second day, 2 on the third, 1 on

the fourth and 1 on the fifth. In no case were acid colonies developed on the plates. All were due to sporulating anaerobes as shown by the corresponding tests with the heated fractions of these samples. All these samples had originally contained *B. coli* according to the Bureau of Sanitary Engineering though in no case in less than 10 cc.

At this stage in the investigation we were ready to adopt the use of 1–100,000 gentian violet in all of the litmus lactose agar plates as a routine procedure in the isolation of *B. coli* from positive presumptive tests, upon the basis of experimental trials with pure cultures of *B. coli* and the hay bacillus. The

TABLE 5

Tests for B. coli and sporulating gas-forming anaerobes in 22 samples of water—using 1–20,000 gentian violet in the presumptive test and 1–100,000 gentian violet in the litmus lactose agar plates

TREATMENT OF SAMPLE	SAMPLE SHOWING GAS IN LACTOSE BROTH	SAMPLES SHOWING ACID COLONIES ON GENTIAN VIOLET LITMUS LACTOSE AGAR	SAMPLES FROM WHICH B. COLI WAS ISOLATED AND IDENTIFIED		
			First trial	Second trial	Total
Unheated	1–20,000 gentian violet, 21	15	15	4	19
	No gentian, violet, 22	14	14	6	20
Heated 56°–60°C.; 30 minutes	1–20,000 gentian violet, 4	2	1*	0	1
	No gentian violet, 22	4	1*	0	1

* Not the same sample.

considerations involved in this change of method have already been discussed fully under the heading of "technic." Twenty-two new samples were tested according to this plan as shown in table 5.

At least 18, or over 81 per cent, of these samples contained anaerobic sporulating gas formers, as judged by the positive tests in the standard presumptive inoculated with heated samples. In 1 case *B. coli* escaped the heating and was isolated and identified as the gas former; in 3 others, Gram-positive-aerobic sporulating gas formers belonging to the hay bacillus group were isolated. The recognition of members of this group

as capable of giving rise to a positive presumptive test in lactose broth is important for these organisms are usually non-lactolytic. Peculiarly these organisms grew, though slowly, upon the gentian violet litmus lactose agar used for plating; their colonies could easily be differentiated from the colon bacillus by the marked manner in which they absorb the dye from the media. In only 1 of the 3 was there a suggestion of acid reaction in the plate and this was anything but marked. It suggests that possibly these organisms produce gas through putrefaction, as most of the anaerobic spore bearers are known to do in the absence of fermentable carbohydrates. It also raises the question as to whether some of the spurious presumptive tests which bacteriologists have been in the habit of ascribing to anaerobic spores may not be due to aerobic spores. This brings us to another interesting point, namely that 16 of the gentian violet litmus lactose agar plates made from these standard presumptive tests with heated water samples not only showed no acid, but were absolutely barren for at least forty-eight hours. It will be remembered that these plates in some of the other series showed mainly, if not entirely, Gram-positive bacilli of the hay bacillus group. The inhibition of this group in this last series of tests is no doubt responsible for the fact that in the two other instances plates showing acid colonies due to streptococci were recorded. We recall that Krumwiede and Pratt (1914) found streptococci and pneumococci somewhat more resistant to gentian violet than some other Gram-positive bacteria.

None of these heated samples however gave positive presumptive tests in the lactose broth with gentian violet. The four positives obtained were from samples of which plates from the standard test were sterile, as mentioned. In one case the fermentation tube was full of gas in twenty-four hours; *B. coli* was isolated from among the acid colonies on the plate. In one other an aerobic, acid forming spore was found to comprise the single colony on the plate. The presumptive test in this instance though showing no gas till the fourth day then developed a tube full, at the same time showing a marked decolorization of the dye. The 2 other positive presumptives showed gas only

after three and four days respectively and failed to give aerobic growth on lactose or plain agar with or without gentian violet; they were found to contain apparently pure cultures of sporulating anaerobes. There has not been time to identify these organisms with certainty, but finding them in the positive presumptive test with gentian violet indicates that for the best results we shall have to increase the concentration of dye somewhat over that with which we have hitherto experimented, although even a concentration of 1–20,000 gives nearly perfect results, as we have shown. A comparison of these cultures, numbers 210, 215 and 216 as to their resistance to gentian violet, in table 1, has shown that they are not unique in this respect.

Among the unheated samples *B. coli* was isolated from both the standard and dye presumptive tests eighteen times. In one sample tested in dye broth another instance of supposed *B. cloacae* turned out to be a mixture of *B. coli* and *B. proteus.*

One sample yielded "attenuated" *B. coli* with difficulty from the dye test and not from the plain lactose broth test. It is safe to say that the organisms could not have been isolated from plain litmus lactose agar without the dye, since the culture in the non-dye tube was heavily overgrown with a member of the hay bacillus group, which did not appear on the plates, however.

Two samples yielded *B. coli* from the standard test and not from the dye test; the gentian violet litmus lactose agar plates made from the last failed on two successive occasions to show aerobic growth. Unfortunately the gas formers, probably dye resistant anaerobes, (though these samples were not the same as those from which we recovered the pure cultures of anaerobes in the heated fractions) were lost before the next subculture was successfully made. We were surprised to find that these last samples were originally heavily contaminated, yielding *B. coli* in 0.001 cc., according to the record of the Bureau of Sanitary Engineering. The result seems to support the idea expressed by Mr. Frank Bachman (1917) that it is such samples from which *B. coli* disappear most readily on standing.

One sample failed to yield *B. coli* in any of our tests; anaerobes were present however, giving positive presumptive tests

in the standard broth, but totally negative in the presence of gentian violet. According to the Bureau of Sanitary Engineering *B. coli* was originally isolated in 20 cc. only and was therefore considered to have died out from the sample.

Again we can call attention to the disproportion between 1st and 2nd successful isolations of *B. coli* favoring the use of gentian violet in the presumptive test, although in this series of tests the total number of samples yielding *B. coli* in the dye test was exceeded by one in the standard test, due to the fortuitous circumstances above mentioned.

TIME OF FIRST APPEARANCE OF GAS

Standard methods permit a negative report on presumptive tests failing to show gas in forty-eight hours. In the beginning of our work we adopted a somewhat longer period, namely five days, in order to isolate as large a proportion as possible of colon bacilli present. It is interesting to note that in no case was *B. coli* isolated from a presumptive test first showing gas after four days. With gentian violet 77 per cent of the tests from which *B. coli* was isolated showed gas within forty-eight hours, without gentian violet, 88 per cent. The figures are not quite representative, however, as we find on analysis that the presence of gentian violet in the presumptive test does not generally delay appearance of gas. The somewhat unfavorable percentage is due almost entirely to a small group of samples tabulated in table 5. These were originally very badly contaminated, but had almost eliminated *B. coli* on standing; if table 5 were excluded the result would stand 88 per cent positive within forty-eight hours with gentian violet, to 87 per cent without. The data for all the tests are tabulated in table 6. The main point seems to be that we are justified in waiting at least four days before calling the presumptive test negative.

The positive tests from which it was impossible for us to isolate *B. coli* are also tabulated, in table 7.

The figures include 1st trials many of which failed to yield *B. coli* but from the subcultures of which in a second presump-

tive test a successful isolation was made. The highest total number of positive tests in this series, from which *B. coli* could not be isolated, consists of course of tests made of heated samples in the standard lactose broth. We feel justified in interpreting these as due mainly to sporulating anaerobes although the figures contain those few cases in which lactolytic aerobic spores were isolated, as do also the few cases showing a test in the presence of the dye. The general tendency of tests not showing *B. coli* is delayed gas production; taking all the tests in table 7 more than 32 per cent occurred after the second day.

A general conclusion from tables 6 and 7 is that the time of appearance of gas gives no certain clue to the nature of the gas former.

AMOUNT OF GAS

Any arbitrary amount of gas required in the presumptive test to be interpreted as a positive test is apt to be misleading, because the presumptive test frequently deals with mixed cultures, and we believe usually containing other gas formers aside from *B. coli*. And by no means most of these produce less than 10 per cent gas in the closed arms; on the other hand many cultures which we can not consider other than members of the colon group produce less than 10 per cent gas and indeed certain coliform bacilli ferment lactose with the formation of acid only. While none of the latter has been so designated in this study because we are concerned with the question of gas formers in the presumptive test, yet the writers are strongly inclined toward Kligler's (1914) view that acid formation is a true criterion of fermentation by *B. coli*. Levene (1916), on the contrary, has held that gas formation is the true evidence of fermentation. Frequently, as Levene suggests, no doubt usually, in the case of *B. coli*, the two phenomena are correlated. In the case of many anaerobes, however, and possibly of some aerobic organisms, gas is produced abundantly even in the absence of fermentable carbohydrates and it is just this which has made the former attempts at classification of the anaerobic spore bearers on the basis of gas formation so confusing.

Because of our skepticism as to the importance of the *quantity* of gas in the presumptive test, complete records were not kept. Since the appearance of the new standard methods, however, we have made numerous observations which need not be tabulated to show that the amount of gas appearing in the presumptive

TABLE 6

First appearance of gas in presumptive tests from which B. coli was isolated

TREATMENT OF SAMPLE	LACTOSE BROTH	DAYS					
		1	2	3	4	5	Total
Not heated	Gentian violet	30	17	4	10	0	61
	No gentian violet	35	18	3	2	0	58
Heated	Gentian violet	1	0	0	0	0	1
	No gentian violet	1	0	0	2	0	3
Total		67	35	7	14		123

TABLE 7

First appearance of gas in presumptive tests from which B. coli was not isolated

TREATMENT OF SAMPLE	LACTOSE BROTH	DAYS					
		1	2	3	4	5	Total
Not heated	Gentian violet	13	10	5	2	3	33
	No gentian violet	29	16	7	4	3	58
Heated	Gentian violet	0	0	2	3	0	5
	No gentian violet	21	31	10	14	3	79
Total		63	57	24	22	9	175

test is of little importance. *B. coli* may frequently be isolated from tubes showing only a trace of gas or tubes full of gas may be due to sporulating aerobes or anaerobes only.

SUMMARY

This report deals with the question of the true interpretation to be placed upon the presumptive test for *B. coli* as a criterion of polluted water. It is pointed out that spurious tests are

frequently due to sporulating organisms of little or no significance from the sanitary viewpoint. These are mainly anaerobic bacilli, but aerobic gas producing spore bearers also occur. Both groups are inhibited by gentian violet in proportion to the concentration, and, whereas *B. coli* is not inhibited by a much greater concentration of this dye, its use for the selective inhibition of the former organism is urged. Tests were made at a concentration of 1–100,000 and 1–20,000 in the presumptive test and at 1–100,000 in a litmus lactose agar plating medium. In all, 65 samples of water, from which *B. coli* had previously been isolated, were tested; 54 were found to contain both sporulating anaerobes and *B. coli*, 1 contained only *B. coli* and no anaerobes, and 10 showed anaerobes only, *B. coli* having disappeared therefrom on standing. The tests showed that gentian violet in the presumptive test not only does not interfere with the isolation of *B. coli* but actually favors it according to the statistical analysis of the results. In the 10 samples set down as containing only anaerobes, presumptive tests were negative in the dye test—positive in the standard test. A most striking feature is the nearly complete inhibition of growth in the case of heated samples tested in dye broth—these containing, with a few exceptions, only sporulating organisms.

Some positive tests were obtained in gentian violet lactose broth from which *B. coli* could not be isolated; barring the possibility that these were due to anaerobic Gram-negative non-sporulating bacilli, it is believed they can be avoided completely by increasing the concentration of the dye. It may be suggested, however, that if one part gentian violet in twenty liters of lactose broth inhibits nearly 95 per cent of the spurious presumptive tests, as inspection of the proportion (4 to 78) of false tests obtained with and without gentian violet in the heated samples shows, it would scarcely be worth while to increase the concentration greatly for the extra 5 or 6 per cent. Even if we analyze the figures in the least favorable manner, i.e., to show the percentage of samples yielding *B. coli* from positive presumptive tests in all our examinations we have the following:

	POSITIVE PRESUMPTIVES	B. COLI ISOLATED	PERCENTAGE
Unheated samples:			
Tested in gentian violet lactose broth	74	61	82.4
Tested in standard lactose broth	85	58	68.2
Heated samples:			
Tested in gentian violet lactose broth	5	1	20
Tested in standard lactose broth	81	3	3.7

Thus while we cannot claim to have progressed to a point where it is safe to dispense with the litmus lactose agar plate and rely wholly upon the presumptive test, we have in the use of gentian violet a method which offers promise of even such a result in higher concentrations than we have used and in the lower concentrations provides a method of eliminating the great majority of spurious tests, obviating the necessity of much futile plating with a consequent saving in time and material.

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A BRIEF NOTE ON THE USE OF GENTIAN VIOLET IN PRESUMPTIVE TESTS FOR B. COLI IN MILK WITH REFERENCE TO SPORULATING ANAEROBES

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In the case of milk, as formerly in the case of water, qualitative bacteriological standards have been preceded by quantitative methods of evaluation, a trend of evolution just opposite to that usual in science. The main reliance has been upon the plain agar plate count though this is now professedly inferior to the lactose agar plate for approximating the total aerobic plate count as recently shown by Sherman (1916). But these plating methods take no cognizance of anaerobes and other bacteria whose particular metabolic requirements happen not to be met by the conditions set, and so the most recent Standard Methods for Bacteriological Analysis of Milk (A. P. H. A. 1916) tend to lay emphasis upon the method of direct microscopic examination, the literature of which has been so adequately reviewed and the technic perfected by Breed and Brew (1916).

While appreciating the fact that the bacterial flora of milk is well known and that qualitative examinations are frequently made when searching for the cause of specific abnormalities in milk we note that such examinations have not as yet become a part of our routine procedure.

In California, however, the recent passage of laws (Cal. 1915) effective October 1, 1916, prohibiting the sale without pasteurization of milk from cows not shown to be free from tuberculosis by the tuberculin test, has suggested the presumptive test for *B. coli* as a criterion of unsuccessful operation of the pasteurizing apparatus, the presence of this organism being

assumed even in milk passably clean. Ayers (1917) has recently pointed out the significance of *large* numbers of *B. coli* in raw milk as indicating temperature conditions suitable for development rather than heavy initial contamination and his viewpoint is confirmed in its essentials by Harding's condemnation (1917) of the bacterial count as an index of cleanliness.

In attempting the use of the presumptive test for *B. coli* as a criterion of unsuccessful pasteurization, Mr. W. H. Stabler, a student in the Department of Veterinary Science, to whom we are indebted for the suggestion, found in a number of cases a situation apparently analogous to that often described in water, namely, gas formation in lactose broth tubes from which no *B. coli* could be isolated. The result was interpreted as due to sporulating anaerobes resistant to the temperature of pasteurization.

Accordingly, after testing the use of gentian violet in presumptive tests for *B. coli* in water (1918), we began testing milk samples under similar conditions, except that 1 cc. of milk was tested in each case, whereas the water tests were made with 10 cc. each. The amount of broth in each tube was 20 cc.

Plates of litmus lactose agar were streaked from tubes showing gas for isolation of *B. coli*. After the first six samples 1 part gentian violet in 100,000 was added to the litmus lactose agar to inhibit the growth of Gram positive spores (most of them non lactose fermenters), through whose spreading proclivities, combined with rather marked proteolytic activities, the acid produced by *B. coli* may be masked, as noted in the case of plates from the presumptive tests of water. A further advantage in the case of milk is that lactolytic cocci, which are notably frequent, are also inhibited, thus further favoring the liklihood of isolated acidifiers proving to be colon bacilli, though there is not much danger of confusing them because of the difference in type of colony. Further details of technic were the same as in the water tests.

The data obtained are summarized in table 1.

Thirty-three samples from ten different sources were tested, 13 of raw milk from tuberculin tested cows, 14 of pasteurized

milk, 2 of certified raw milk, and 4 not labelled. We do not wish at this time to lay great emphasis upon the findings of *B. coli* as distributed among these classes further than to say that a surprisingly large number of pasteurized samples, namely, 10 out of 14, contained *B. coli*.

What we do wish to emphasize is, (1), that no evidence was gained of the survival of anaerobes after heating to 56–60° for thirty minutes as shown by the presumptive test in lactose broth, with or without gentian violet; (2), that the use of gentian violet does not prevent the isolation of *B. coli* from unheated samples. Indeed 3 samples gave positive presumptive tests

TABLE 1

TREATMENT OF SAMPLE	SAMPLES SHOWING GAS IN LACTOSE BROTH	SAMPLES SHOWING ACID COLONIES IN LACTOSE AGAR		SAMPLES FROM WHICH B. COLI WAS ISOLATED AND IDENTIFIED
		No gentian violet	1–100,000 gentian violet	
Not heated	1–20,000 gentian violet, 23	5		2
			18	18
	No gentian violet, 20	3		0
			17	15
Heated 56°–60° thirty minutes*	1–20,000 gentian violet, 1		1	1
	No gentian violet, 1		1	1

* Three samples not tested.

with gentian violet lactose broth and negative tests with plain lactose broth; in only one of these was *B. coli* isolated and the presumptive test was negative up to the fourth day. In the other two cases *B. coli* was not isolated from the sample; had gentian violet not been omitted from the lactose agar plate in these two cases *B. coli* might have been isolated as in the other case with its use. In no case did a sample yielding *B. coli* in the presumptive test without gentian violet fail to yield this organism from the presumptive test with gentian violet.

We note that in 5 positive presumptive tests in gentian violet lactose broth from which streaks made on plain lactose agar plates

showed acid colonies, *B. coli* could be isolated but twice. The acid colonies in each of these cases proved to be gram positive cocci. In 3 samples, plates of which were acid after streaking from positive presumptive tests in plain lactose broth, gram positive cocci were responsible in each case for the acid; gas forming aerobes could not be isolated, but from one came a gram negative coliform bacillus giving acid on litmus lactose agar and not liquefying gelatin. This and one other sample of the three yielded *B. coli* from the presumptive test in gentian violet lactose broth plated on plain litmus lactose agar. From 18 samples tested in lactose broth with 1–20,000 gentian violet and streaked out on gentian violet litmus lactose agar *B. coli* was isolated in each case; from 17 samples tested in plain lactose broth and streaked out on gentian violet litmus lactose agar *B. coli* could not be isolated in two cases. In one of them only a large Gram positive bacillus, acidifying the plate and apparently not completely inhibited by the gentian violet at a concentration of 1–100,000, was isolated; it did not produce gas from lactose and the subculture from the first presumptive test into a second showed that we had lost the gas former; *B. coli* was isolated from the presumptive test of this sample in lactose broth with 1–20,000 gentian violet. In the other only streptococci appeared on the plate, which was acidified by them; they too were not inhibited by the weaker concentration of gentian violet in the plate and the gas former was lost when the second presumptive test was made; *B. coli* was isolated from the presumptive test on gentian violét lactose broth in this case also.

The single heated sample showing gas in the presumptive test yielded *B. coli* from the lactose broth with, as well as without, gentian violet. The organism had apparently been able to survive the heating. We cannot of course be certain that sporulating anaerobes were absent from this specimen any more than we can be sure that they were absent from the unheated specimens yielding gas in the presumptive test.

From a comparison of our work on water with that on milk we are inclined to conclude that the so-called "attenuated" *B. coli* is less frequent in milk than in water; a larger proportion

of successful isolations came from the first presumptive test. We are not at all sure what constitutes "attenuated" *B. coli*, whether these are organisms that have temporarily lost their ability to ferment lactose or whether they have lost only their ability to ferment lactose aerobically but not anaerobically, that is to say, under a reduced oxygen pressure. In water work we and others have frequently isolated aerobic non acidifying colonies from litmus lactose agar plates which form gas and acid in the fermentation tube, though usually slowly; this seems to point to the latter condition. In our milk studies no such cases were encountered, due we believe to the fact that colon bacilli recovered from milk on lactose agar are in full possession of their lactolytic power because of the recent activity of that function in the milk itself.

Our main conclusion is that while we are not justified as yet in claiming that the addition of gentian violet to lactose broth will enable us to say in *every* case that *B. coli* is present when gas is formed, yet its use certainly does not interfere with the demonstration of *B. coli*; indeed it distinctly favors such demonstration. Further, the use of 1–100,000 gentian violet in the litmus lactose agar plate does not interfere with the growth or acid producing properties of the organism; rather it eliminates to a large degree the masking of acid produced by *B. coli*, through the inhibition of alkali forming, proteolytic, non-lactolytic, Gram positive sporulating bacilli of the hay bacillus group, not to mention the advantage of preventing spreading growths due to these organisms. Moreover the inhibition of acidifying cocci is of distinct advantage in the search for *B. coli*.

We are frankly surprised not to have demonstrated sporulating anaerobes in milk heated for thirty minutes to 56 to 60°C. It may well be that larger samples, say 10 cc., such as we have used in our water examination would give positive results. Another explanation may be mentioned tentatively, namely, that the presence of lactose in the milk has caused lactolytic sporulating anaerobes to vegetate so that they would be as susceptible to heating as *B. coli*, since various investigators such as Simonds (1915), Hibler (1908), and others, as well as our

own experience have taught that the presence of fermentable carbohydrates inhibits sporulation of anaerobic bacteria. Aside from these forms, however it is to be expected that certain sporulating anaerobes incapable of fermenting lactose would gain access to milk and, theoretically at least, there should be no inhibition of sporulation in their case. This particular phase of the problem will have to be attacked in another way.

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ON THE VALUE OF THE PETROLEUM-ETHER METHOD FOR THE ISOLATION OF B. TYPHOSUS FROM FECES

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The use of petroleum-ether in the isolation of typhoid bacilli from feces was suggested by Bierast (1914). He added a finger's breadth of petroleum to a fecal suspension in broth and shook the mixture at intervals. After allowing this to stand in a cool place for sixteen hours, he plated the sediment or some of the broth from the bottom of the container. He reported that 2 out of 23 specimens were positive only by this method. The work was interrupted because he developed typhoid fever. Jaffe (1915) had favorable results with 24 specimens, as follows:

NUMBER	DIRECT PLATING	AFTER SHAKING, ETC.
5	−	−
5	+	+
0	+	−
4	−	+

He calls attention to the danger of the method and reports a case of typhoid fever due to its use.

Hall (1915) working with the method found that the activity of the different fractions obtained by the distillation of crude petroleum varied inversely with the boiling point. He found that the fraction with a boiling point about 40°C. gave the best results. This fraction would contain a large proportion of pentane ($C_5 H_{12}$) and for convenience we shall refer to it as such.

Hall modified the Bierast procedure as follows. To a moderately heavy suspension of feces in broth is added one-half its

volume of pentane. Close the bottle tightly with a rubber stopper and shake for one-half hour in a shaking machine. The container is then allowed to stand for one and one-half to two hours at room temperature, the bottom fluid or sediment removed with a pipette and plated. His results may be summarized as follows;

SPECIMENS EXAMINED	DIRECT PLATING	PENTANE SHARING
69	Positive, 13	Positive, 13
	Positive, 1	Negative, 1
	Negative, 7	Positive, 7
	Negative, 48	Negative, 48

Thus, while one specimen positive on direct plating was negative after shaking, 7, or 33 per cent of the total positives, were positive only after shaking.

These results were so favorable that we investigated the method. Unless otherwise stated, we have followed the method of Hall.

Our first attempts were made with stools from typhoid carriers and with greatly diluted broth cultures of recently isolated typhoid strains. The shaking was for one-half hour, and after shaking, the emulsions were allowed to stand for two hours before plating on the Endo medium. The results may be roughly illustrated by the following examples:

MATERIAL	DIRECT PLATING	AFTER SHAKING
Stool suspension	Positive—5 per cent typhoid	1 typhoid colony
Stool suspension	Positive 4,000 total colonies; 75 per cent typhoid	100 total colonies; 90 per cent typhoid
Stool suspension	Positive 5,000 total colonies; 5 per cent typhoid	200 total colonies; 1 per cent typhoid

Broth suspensions of several cultures (pure) yielding by direct plating from 400 to 5000 colonies per plate, after shaking, and then plating an equivalent amount of material, gave no colonies.

While, with the one stool cited above, there was a relative increase in the proportion of typhoid to total flora, it would

obviously be dangerous to expose stools less rich in typhoid to this marked absolute decrease in typhoid colonies. We next tried shortening the time of shaking, and found that in no case did the proportion of typhoid increase on shaking longer than ten minutes, whereas the total number recovered frequently took an alarming drop, and in some cases none was recovered after thirty minutes. It was also determined at this time, that more typhoid bacilli were viable after one than after two hours' standing. The results with this modified technique—that is, time of shaking reduced to ten minutes, and of standing to one hour—may be summarized briefly as follows.

TOTAL EXAMINED	DIRECT PLATING	AFTER SHAKING
12	Positive, 10	Positive, 10
	Positive, 1	Negative, 1
	Negative, 1	Positive, 1

These stools were quite rich in typhoid. Of those in which bacilli were found both before and after shaking, the percentage of typhoid to total flora was increased in three and decreased in seven cases. In 3 of the 7, less than 10 per cent of the number of colonies on the direct plates developed after shaking. In the case of the 3 specimens showing relative increase, 2 of the 3 specimens showed loss of typhoid bacilli, in 1 case to 3 per cent of the original number. The stool which gave positive results only on direct plating had 75 per cent of typhoid (total colonies 900) and yielded 150 non-typhoid colonies after shaking. That in which bacilli were found only after shaking had 6000 colonies on two badly diffused direct plates, which were reduced on shaking to 400, of which 20 were typhoid. In other words, the tendency, even with the reduction of exposure, is to a rapid reduction of the number of typhoid bacilli with which a relative increase compared with the accompanying flora is not necessarily associated.

While these experiments were under way, we conducted a number of tests with pure cultures of recently isolated strains of typhoid, designed to develop a technique more favorable than

that which we were employing. First, we determined that an emulsion of typhoid in broth was not more susceptible to the action of pentane than a similar emulsion in either a normal stool suspension, or in the same suspension sterilized by steaming for one-half hour. Thus:

Summary of reactions of several strains: shaken ten minutes, stood one hour

Direct plating	*After shaking*
In broth 2500 colonies	0
In stool suspension 2500 colonies, 95 per cent typhoid	0
In sterilized stool suspension 2500	0

On attempting to find the approximate percentage of typhoid bacilli which would survive shaking alone, we found wide variations between individual strains. With some, 250 colonies per drop were completely lost on shaking; with others, the numerical reduction varied from 40 to 98 per cent. With this reduction in numbers, there was frequently a concomitant reduction in the size of the colonies developing after shaking. It was thought that the physical effect of shaking might be responsible, but on shaking without pentane (with equivalent saline dilution) practically no reduction occurred, whereas, in this case, 5000 colonies per loop were killed by pentane. It was thought that the bacilli might be contained within the supernatant pentane rather than in the subjacent broth, but culture from the pentane in two cases where the broth yielded from 3 to 25 colonies per drop, showed it to be sterile.

We then investigated more accurately the effect of standing after shaking. The results with several strains were:

DIRECT PLATING	IMMEDIATELY AFTER SHAKING	STOOD 15 MINUTES	STOOD 30 MINUTES	STOOD 45 MINUTES	STOOD 1 HOUR
5,000*	3,000	3,000	2,000	2,000	1,800
3,000	600	160	65	27	25
1,400	400	21	12	12	3

* Strain (in other tests) shows unusual resistance to pentane.

In a final series of stool examinations, small amounts of carrier stool were added to normal stools so as to give mixtures poor in

typhoid. Obviously, it is only with this class of material that the method, if of value, will aid in diagnosis. In order to determine more accurately the initial number of typhoid bacilli, we incorporated the use of the brilliant green medium described by Krumwiede, Pratt and McWilliams (1916), which has proved extremely successful in the isolation of typhoid bacilli from feces. As preliminary tests had shown that in stools as well as in broth cultures, the typhoid bacilli were more or less rapidly lost on standing after the shaking was over, we plated immediately after shaking ten minutes with pentane, and obtained the following results.

TOTAL EXAMINED	DIRECT ENDO	DIRECT GREEN-DYE	ENDO AFTER SHAKING
50	Positive, 21 Negative, 7 Negative, 9 Negative, 13	Positive, 21 Positive, 7 Positive, 9 Negative, 13	Positive, 21 Positive, 7 Negative, 9 Negative, 13
Total positives	21	37	28
Percentage positive	42.0 per cent	74.0 per cent	56.0 per cent
Percentage positive compared with total number positive	56.7 per cent	100.0 per cent	75.6 per cent

These results indicate that the method with very short exposure has some value as compared with the direct plating on a non-restraining medium (Endo) alone. The general tendency to reduction in the number of typhoid bacilli, not necessarily associated with a markedly greater decrease in the fecal flora, was again observed in this series. On the whole, the data presented indicate that the method is not a reliable one for the relative enrichment of typhoid bacilli in stools. Compared with direct plating on a differential medium (brilliant green agar) the method is evidently inferior.

A report by Schuscha (1916) recently available only in an abstract indicates that his results with the method have not been more satisfactory than with direct plating.

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We might add that the method of pentane shaking is unsuited to routine laboratory work. The pentane itself is highly inflam-

mable, and the containers are protected from leaking during agitation in the machine only with difficulty. Two workers abroad have developed typhoid fever while using the method and we feel that there is a large element of danger in its use because of the tendency for the contents of the bottles to blow out when opened, due to the highly volatile pentane.

CONCLUSIONS

The petroleum-ether method for the detection of *B. typhosus* has no advantage over direct plating on a medium which restrains the growth of the associated fecal types. With short periods of exposure, it may be successful where direct plating on Endo alone fails. There is a strong tendency to reduction of the number of *B. typhosus*, not necessarily associated with a greater reduction of the accompanying fecal bacteria. The danger of infection to those using the method, however, is sufficiently serious to warrant its condemnation, especially as it offers no advantage over direct plating on differential restraining media.

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BACTERIAL NUTRITION: FURTHER STUDIES ON THE UTILIZATION OF PROTEIN AND NON-PROTEIN NITROGEN

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It has been demonstrated by independent investigators (Bainbridge, 1911, and Sperry and Rettger, 1915) that bacteria are unable to bring about the decomposition of native or unchanged proteins by direct action. These proteins can serve as food material for bacteria only through the agency of cleavage-producing substances like acids, alkalies and proteolytic enzymes. Even the most active gelatin-liquefying and putrefactive organisms die from inanition in a medium which contains egg albumin, serum albumin or the vegetable protein, edestin, as the only possible source of nitrogen.

It has been shown further by Rettger, Berman and Sturges (1916) that egg albumin which has been coagulated by heat still retains its resistance to direct bacterial action, and that the proteoses and the higher or more complex polypeptids or peptone fractions of Witte's "peptone" apparently show the same indifference to microbic influence.

The existence of an erepsin-like enzyme in certain bacteria was clearly indicated in the experiments of the writers (1916) which have been conducted on nitrogen utilization in Witte's peptone. The peptolytic enzyme elaborated by the Coli-typhi-paratyphi group and by other gelatin-non-liquefying organisms studied is decidedly weak, and differs further from erepsin of the animal body by its inability to attack casein. At least two weeks were required in the earlier tests to bring about a noticeable reduction in the amount of biuret-giving substances in Witte's

peptone, and even after four weeks of incubation the decrease was small, with one exception.

The work upon which this paper is based was a continuation of the investigations to which reference has been made, and dealt with the following topics:

a. The behavior of bacteria towards coagulated egg albumin.
b. The utilization of different brands of commercial peptone.
c. The utilization of "purified" proteose.
d. The utilization of gelatin and casein.
e. The utilization of the products of Witte's peptone and of casein obtained by partial digestion with trypsin.

THE BEHAVIOR OF BACTERIA TOWARDS COAGULATED EGG ALBUMIN

The results obtained were in strict accord with those of the earlier experiments. A number of additional organisms were employed in the later tests, as for example *Pseudomonas fluorescens*, *Bacillus cloacae* and new strains of *Proteus vulgaris*, *Bacillus subtilis* and *Bacillus coli*.

But one conclusion can be drawn from the results of the combined tests. Coagulated egg albumin, like the unheated and unchanged proteins previously studied, resists the action of even the most active proteolytic bacteria, and can be utilized as food only after at least partial disintegration or cleavage by enzymes or other protein-destroying agents. In all of these experiments the methods employed were the same as those described by Sperry and Rettger (1915).

The individual cell, whether animal, plant or bacterial, requires simple forms of nitrogen, at least until it may produce its own enzymatic substance. The nourishment of bacteria is, as Distaso (1916) has well stated, a passive one, and depends upon substances which can readily pass through the cell walls and be absorbed by the protoplasm. This view is further supported by the experiments which follow.

THE UTILIZATION OF DIFFERENT BRANDS OF COMMERCIAL PEPTONE

Laboratory culture media with few exceptions contain "peptone" as their main nitrogenous ingredient. At the present time various brands of this soluble protein and amino acid complex are available in the open market. The most popular and best known is the Witte product, which until quite recently was used almost exclusively. Chemical analyses indicate that this product has been incorrectly labelled. It has been more than thirty years since Kühne and Chittenden (1884) published their results of the analysis of Witte's peptone. They found the preparation to be rich in albumose. The uniformity of this brand as to composition has made it the universal standard for many years.

At the present time several American brands of peptone are on the market which bid fair to take the place of the Witte product, but none of which have as yet attained the individual prominence of the foreign brand. Chemical tests have shown that the American brands differ to a considerable extent in their composition.

Several methods are available for the study of "peptone" utilization. A brief review will show, however, how far from adequate, with few exceptions, these methods are, and how they may readily lead to wrong conclusions.

Abderhalden, Pincussohn and Walther (1910) employed optical methods in their investigation of the action of certain pathogens on peptone. Since the optical properties of the products of decomposition may have a neutralizing effect, the use of such a test is open to error. The presence of meat extract in a medium may also interfere.

Crabill and Reed (1915) studied the biochemical activities of microörganisms by pouring the test media into Petri dishes and inoculating them with the bacteria under investigation. The digestion and utilization of the medium could thus be determined by direct observation of the plates. Like the auxanographic method of Eijkmann (1901), this is a qualitative procedure and consequently not suitable for the present problem.

The quantitative determination of ammonia production in nutrient broth has been extensively made use of in estimating the comparative ability of various groups of bacteria to attack protein. Among the staunch supporters of this method are Kendall and his co-workers. Caution is necessary in drawing conclusions from the results of this test, for an increase in the ammonia content of a medium does not necessarily indicate that the protein has been attacked. There are other sources of ammonia in nutrient bouillon besides albumoses and peptones, as for example amino acids and extractive substances.

The biuret test was employed in these experiments, since experience in the earlier work had proven its merit. It is of value in demonstrating the disappearance by digestion or otherwise of protein in a fluid medium. Peckham (1897) used this test to show that *B. coli* is unable to utilize completely the protein present in ordinary nutrient media. Vernon (1904) was the first, however, to demonstrate the value of the biuret method as a quantitative measure of peptone digestion, in his study of the action of erepsin upon commercial peptone.

The formol titration method of Sörensen (1908) was employed in conjunction with the biuret test. This method has already been applied to bacteriological problems. The titration figure is a measure of the amount of primary amino acids present in a medium. Rice (1915) claims to have found the Sörensen more satisfactory than the Van Slyke method for the determination of free amino nitrogen.

The biuret test was employed in essentially the same manner as described by Vernon (1904) and as given in a previous paper from this laboratory (1916). The Sörensen method was conducted as follows. Five cubic centimeters of the medium to be tested were mixed with 50 cc. of tap water and neutralized with N/20 NaOH or HCl. Two cubic centimeters of neutral formaldehyde were then added and the solution again neutralized, with phenolphthalein as the indicator.

In the tables the formol titration values are recorded as the number of cubic centimeters of N/20 NaOH required for 100 cc. of the test medium. In the column under 'Reaction' a similar plan has been followed.

The first part of this investigation involved a study of the utilization of Witte's peptone by different types of bacteria. In later experiments other brands of peptone were investigated. The test media were, as a rule, simple nutrient broths such as are used for routine work in the laboratory. These solutions were always transferred to large test tubes in amounts of 10 cc. The water lost by evaporation was accurately replaced after the incubation period, and preliminary to the testing.

Witte's peptone

In tables 1, 2 and 3 are recorded the results of the experiments on the utilization of Witte's peptone. It will be seen that each of the organisms studied is capable of reducing the amount of biuret-giving substances, though for the gelatin-non-liquefying organisms at least a week's incubation is required to show any

TABLE 1

Showing the utilization of Witte's peptone

ORGANISMS	REACTION	SÖRENSEN TEST	BIURET TEST
Control	12.0	18.0	1.0
Subtilis group, average 3 members	*3.3*	49.0	0.0
B. prodigiosus	*4.0*	42.0	0.0
P. vulgaris, average 2 strains	*5.0*	52.0	0.0
P. mirabilis	*4.0*	62.0	0.30
Staph. aureus, 3 strains	*2.0*	47.0	0.55
Staph. albus	*4.0*	52.0	0.50
B. cloacae	*4.0*	52.0	0.40
B. typhi	2.0	32.0	0.65
B. paratyphi A	0.0	32.0	0.70
B. coli, average 4 strains	1.0	43.0	0.75
B. diphtheriae, average 2 strains	5.0	37.0	0.60

Medium employed: 1 per cent peptone, 0.25 per cent Liebig's beef extract, and 0.5 per cent NaCl. Incubation at 30°C. for three weeks.

Note: When results with similar organisms do not differ widely they are averaged for the sake of brevity.

The standard biuret figure for the control is given as 1.0 in all of the tables. According to this system a reduction of 40 per cent in the amount of biuret-giving substances is recorded as 0.60, and one of 100 per cent as 0.0.

Reaction of culture fluids is given in the first column. The results are stated in terms of N/20 acid or alkali. The italic figures denote alkaline reaction.

diminution in these substances. Most of the gelatin liquefiers were able to utilize the proteins completely within a period of three weeks. *B. subtilis*, *B. cereus*, *B. prodigiosus* and *Proteus vulgaris* were especially active in this respect. On the other hand, the non-liquefiers exerted but a weak peptolytic action, the average amount of biuret-giving substances acted upon being less than 30 per cent of the total. Among the gelatin

TABLE 2

Weekly tests showing utilization of Witte's peptone

ORGANISMS	BIURET TEST				SÖRENSEN TEST			
	1 week	2 weeks	3 weeks	4 weeks	1 week	2 weeks	3 weeks	4 weeks
(1) Without beef extract								
Control	1.0	1.0	1.0	1.0	16.0	16.0	16.0	16.0
B. subtilis	0.9	0.0	0.0	0.0	62.0	104.0	110.0	90.0
Staph. aureus	1.0	0.9	0.9	0.8	20.0	34.0	40.0	46.0
B. cloacae	1.0	0.8	0.8	0.8	28.0	36.0	44.0	52.0
B. typhi	1.0	1.0	0.9	0.9	14.0	22.0	26.0	32.0
B. coli, average 2 strains	1.0	0.8	0.8	0.8	20.0	31.0	42.0	52.0
Medium: 1 per cent peptone, and 0.5 per cent NaCl.								
(2) With beef extract								
Control	1.0	1.0	1.0	1.0	24.0	24.0	24.0	24.0
B. subtilis	0.85	0.3	0.0	0.0	84.0	108.0	130.0	112.0
Staph. aureus	0.9	1.0	1.0	0.7	34.0	48.0	54.0	76.0
B. cloacae	0.9	0.7	0.6	0.7	60.0	82.0	92.0	78.0
B. typhi	1.0	1.0	0.9	0.9	26.0	34.0	42.0	48.0
B. coli, average 2 strains	1.0	1.0	0.8	0.75	30.0	44.0	54.0	54.0
Medium: 1 per cent peptone, 0.25 per cent Liebig's beef extract and 0.5 per cent NaCl.								

liquefiers *Staphylococcus aureus* and *St. albus* and *B. cloacae* also were comparatively weak.

Table 3 shows that different salts used in medium B do not affect the course of decomposition of the peptone, as compared with the plain nutrient broth.

The small decrease in the biuret-giving substances during the first week of incubation, brought about by the gelatin liquefiers,

as seen in table 2, is of special interest, and would indicate that even strongly proteolytic organisms utilize the simpler nitrogenous substances during their earlier development, thus sparing the more complex substances until the former are exhausted and until abundant enzyme production has taken place.

The formol titration results are of much interest in connection with the other figures. It is quite apparent that an increase in

TABLE 3

Showing utilization of Witte's peptone with and without the addition of certain inorganic salts.

ORGANISMS	MEDIUM A*		MEDIUM B†	
	Biuret test	Sörensen test	Biuret test	Sörensen test
Control	1.0	10.0	1.0	14.0
B. subtilis	0.0	52.0	0.0	48.0
P. vulgaris	0.0	58.0	0.0	46.0
Staph. aureus, average 3 strains	0.65	35.0	0.65	37.0
B. cloacae	0.5	46.0	0.5	50.0
B. typhi	0.75	22.0	0.7	22.0
B. paratyphi A	0.7	22.0	0.7	30.0
B. paratyphi B	0.85	20.0	0.9	24.0
B. bronchisepticus	0.85	12.0	0.9	14.0
B. coli, average 4 strains	0.75	22.0	0.75	27.0
B. diphtheriae, average 2 strains	0.7	23.0	0.75	28.0

* Medium A: 0.5 per cent peptone, 0.25 per cent Liebig's beef extract and 0.5 per cent NaCl.

† Medium B: Same as A plus 0.1 per cent K_2HPO_4, 0.02 per cent $MgSO_4$ and 0.01 per cent $CaCl_2$.

Note: Incubation at 30°C. for three weeks.

the titration value does not necessarily run parallel with the results of the biuret tests.

Other brands of commercial peptone

The other peptones tested were all of American manufacture, namely the Digestive Ferments Company, Parke, Davis and Company, Armour, Eimer and Amend, and the Arlington Chemical Company (aminoids) brands. The results of these experiments are given in tables 4, 5, 6, 7, and 8.

A marked difference in the degree of utilization of the protein material in these different products was observed. The Digestive Ferments Company peptone (Difco) showed approximately the same degree of resistance to bacterial attack by the gelatin-non-liquefying bacteria and the intermediate group of organisms (*Staph. aureus* and *Staph. albus* and *B. cloacae*, as the Witte. On the other hand, "aminoids" and the Eimer and Amend product seem to have undergone the greatest amount of degradation of protein or the more complex biuret-giving substances in the process of manufacture, according to the biuret reduction figures. The "aminoids"[1] brand was reduced by *B. coli*, *B. typhi* and

TABLE 4

Showing the utilization of Digestive Ferments Company peptone

ORGANISMS	REACTION	SÖRENSEN TEST	BIURET TEST
Control	16.0	32.0	1.0
Subtilis group, 3 strains	7.5	64.0	0.0
B. prodigiosus	8.0	62.0	0.0
P. vulgaris, 3 strains	5.0	50.0	0.2
Staph. aureus and *albus*, 4 strains	1.5	50.0	0.77
B. typhi and *B. paratyphi A* and *B.*	2.0	44.0	0.73
B. coli, 4 strains	1.5	44.0	0.7
B. diphtheriae, no. 8	8.0	42.0	0.75

Medium: 0.5 per cent peptone, 0.25 per cent beef extract, 0.5 per cent NaCl. Incubation at 30°C. for three weeks.

B. paratyphi from a biuret value of 1.0 to 0.3 and 0.4, and by *B. diphtheriae* to 0.1 and 0.3, as compared to 0.4 and 0.5 for the Eimer and Amend product. The remaining brands occupy an intermediate position between these two extremes.

The experimental evidence at hand indicates that most of the nitrogen present in commercial peptones is in a form too complex to be utilized directly. *B. subtilis*, *B. prodigiosus*, *P. vulgaris* and the closely related forms are able to digest the different peptones completely, whereas those organisms which are not

[1] Two brands of "aminoids" have been used in this laboratory. The one contains an appreciable amount of biuret-giving substances, while the other is biuret-free. The former was employed in these experiments.

known to elaborate proteolytic or gelatin-attacking enzymes, as for example *B. coli* and *B. typhi*, exert but little influence on the biuret-giving substances of some of the peptones, particularly Witte's.

Commercial peptones are now regarded as being a mixture of polypeptids of all degrees of complexity. It is probable that the peptone fraction of these products contains polypeptids varying in composition from the simplest dipeptids to those which are relatively very complex. The former are more easily utilized as food by the bacteria than the higher or more permanent forms,

TABLE 5

Showing the utilization of Parke, Davis and Company peptone

ORGANISMS	REACTION	SÖRENSEN TEST	BIURET TEST
Control	20.0	28.0	1.0
Subtilis group, 3 members	*3.5*	62.0	0.0
B. prodigiosus	*6.0*	48.0	0.0
P. vulgaris, 2 strains	*3.0*	56.0	0.0
P. mirabilis	10.0	48.0	0.7
Staph. aureus and *albus*, 4 strains	7.5	53.5	0.6
B. cloacae	*2.0*	54.0	0.4
B. typhi and *B. paratyphi A*	*4.0*	54.0	0.6
B. coli, 4 strains	*2.0*	52.0	0.6
B. diphtheriae, 2 strains	6.0	54.0	0.5

Medium: 0.5 per cent peptone, 0.25 per cent beef extract, 0.5 per cent NaCl. Incubation at 30°C. for three weeks.

and hence disappear from solutions first.[2] As even some of the very simple polypeptids are known to give the biuret color reaction, we may find here a possible explanation of the slow, gradual but limited reduction in the biuret figures which the non-proteolytic organisms studied brought about. It is the amino acids and other simple nitrogenous substances, however, to which commercial peptone chiefly owes its value as food for bacteria.

It has been shown repeatedly that meat extract in nutrient broth, etc., contains valuable food substances, and that it is for

[2] Sasaki (1912) has shown that simple polypeptids are attacked by bacteria.

this reason a very important ingredient of the ordinary laboratory media. The older belief in a mere "stimulating effect" of meat extract on bacteria is unscientific and untenable. The real food value lies in the amino acids (primary and secondary) and in the so-called "extractives, kreatin, kreatinin, hypoxanthin, etc."

The behavior of *St. aureus*, *St. albus* and *B. cloacae* in the peptone was surprising. These gelatin-liquefying organisms were able to attack the biuret-positive substances to only a

TABLE 6

Showing utilization of Armour's peptone

ORGANISMS	REACTION	SÖRENSEN TEST	BIURET TEST
Control	20.0	30.0	1.0
Subtilis groups, 3 members	5.0	60.0	0.0
B. prodigiosus	4.0	54.0	0.0
P. vulgaris, 2 strains	1.0	56.0	0.0
P. mirabilis	8.0	66.0	0.4
Staph. aureus and *albus*, 4 strains	7.0	60.0	0.5
B. cloacae	2.0	56.0	0.5
B. typhi, and *B. paratyphi A* and *B*	5.5	50.0	0.65
B. coli, 4 strains	3.5	52.0	0.57
B. diphtheriae, 2 strains	7.0	54.0	0.5

Medium: 0.5 per cent peptone, 0.25 per cent beef extract, 0.5 per cent NaCl. Incubation at 30°C. for three weeks.

slightly greater extent than the non-liquefiers. This observation is in accord, however, with those of Malfitano (1903) who pointed out a distinct difference between the albumolytic and the gelatinolytic properties of an organism. In his work with *B. anthracis* he showed that the addition of chloroform influenced the albumin-digesting, but not the gelatin liquefying property. A further review of the literature (Mavrojannis, 1903, and Jordan, 1906) justifies the conclusion that the ability of an organism to liquefy gelatin is no indication of its proteolytic power.

TABLE 7

Showing the utilization of Eimer and Amend peptone

ORGANISMS	REACTION	SÖRENSEN TEST	BIURET TEST
Control	20.0	24.0	1.0
Subtilis group, 3 members	6.0	49.0	0.0
B. prodigiosus	2.0	48.0	0.0
P. vulgaris, 2 strains	7.0	42.0	0.0
P. mirabilis	14.0	42.0	0.3
Staph. aureus and *albus*, 4 strains	12.0	38.0	0.48
B. cloacae	8.0	38.0	0.4
B. typhi, and *B. paratyphi A* and *B*	7.0	35.0	0.45
B. coli, 4 strains	8.0	37.5	0.43
B. diphtheriae, 2 strains	10.0	39.0	0.43

Medium: 0.5 per cent peptone, 0.25 per cent beef extract, 0.5 per cent NaCl. Incubation at 30°C. for three weeks.

TABLE 8

Showing the utilization of "aminoids" of Arlington Chemical Company

ORGANISMS	REACTION	SÖRENSEN TEST	BIURET TEST
Control	20.0	42.0	1.0
Subtilis group, average 3 members	4.0	57.0	0.0
B. prodigiosus	4.0	54.0	0.0
P. vulgaris, 2 strains	3.0	46.0	0.0
P. mirabilis	12.0	64.0	0.0
Staph. aureus, 3 strains	9.0	59.0	0.3
Staph. albus	10.0	64.0	0.3
B. cloacae	4.0	46.0	0.25
B. typhi, and *B. paratyphi A* and *B*	2.5	56.0	0.3
B. coli, 4 strains	1.0	51.0	0.35
B. diphtheriae	9.0	52.0	0.2

Medium: 0.5 per cent aminoids, 0.25 per cent beef extract, 0.5 per cent NaCl Incubation at 30°C. for three weeks.

THE UTILIZATION OP PURIFIED PROTEOSE

Proteose was obtained from commercial "peptone" by salting out with ammonium sulphate. The first precipitate was dissolved in water and the proteose reprecipitated with the sulphate. After dialysis for several days the proteose solution was converted into a test medium by the addition of Liebig's

extract and sodium chloride. The amounts of the different substances in solution are stated in the following tables.

Another method of at least partial purification of proteose consisted in dialyzing a concentrated solution of "peptone." It was to be expected that the simpler biuret-giving substances which in their aggregate constitute what may be called the peptone fraction of the commercial "peptone" would pass through the parchment membrane, leaving the more complex ingredients, the proteose portion in particular, behind. The process of dialysis was allowed to continue for from six to eight days. In order to prevent bacterial decomposition of the dialysate it

TABLE 9

Showing the utilization of "purified" proteose

ORGANISMS	REACTION	SÖRENSEN TEST	BIURET TEST
Control	6.0	10.0	1.0
B. subtilis and *B. ramosus*	*5.0*	78.0	0.0
B. prodigiosus	*10.0*	68.0	0.0
P. vulgaris, 2 strains	14.0	10.0	0.9
P. mirabilis	16.0	12.0	1.0
Staph. aureus and *albus*, 4 strains	11.0	11.0	0.95
B. cloacae	2.0	22.0	0.95
B. typhi and *B. paratyphi A*	19.0	11.0	1.0
B. coli, 2 strains	12.0	12.0	1.0
B. coli, 1 strain	20.0	10.0	0.95

Medium: 0.8 per cent proteose, 0.25 per cent beef extract, 0.5 per cent NaCl. Incubation at 30°C. for three weeks.

was boiled for a short period each day, and the water in the jar was frequently changed.

The experiments with the proteose media were conducted in the same manner as those in which the commercial peptones were employed. The results of the first series of tests are recorded in table 9. The beef extract was added to encourage bacterial development. Only those culture flasks which gave positive evidence of growth were used.

B. subtilis, *B. ramosus* and *B. prodigiosus* completely destroyed the proteose, as shown by the biuret test. *Proteus vulgaris* and *P. mirabilis* produced only a slight change, owing to their slow

development. The beef extract was not sufficient to supply the initial needs of these organisms for enzyme production. The proteus group is known to find poor growth conditions in synthetic media, particularly the Uschinsky medium. The staphylococcus forms affected the biuret-giving substances but very little, if at all. The members of the Coli-typhi-paratyphi group, furthermore, were unable to attack the purified proteose, during the entire incubation period of three weeks. The Sörensen values strongly support those of the biuret test.

An experiment was conducted to determine whether proteose is attacked in a medium in which it furnishes the sole source of nitrogen. The test solution consisted of approximately 0.25

TABLE 10

Showing the behavior of bacteria in dialyzed Witte's peptone

ORGANISMS	IMMEDIATE	AFTER INOCULATION				REACTION	SÖRENSEN TEST	BIURET TEST
		24 hours	48 hours	6 days	2 weeks			
B. subtilis	11	6	2	2	0	2.0	2.0	1.0
B. prodigiosus	10	x*	x	x	x	2.0	8.0	0.0
B. typhi	16	1	1	2	1	2.0	2.0	1.0
B. coli	16	1	1	1	1	2.0	2.0	1.0
Control						2.0	2.0	1.0

Medium: 0.25 per cent "proteose," 0.5 per cent NaCl. Chemical tests made after incubation of three weeks.

* x indicates too many colonies to count.

per cent proteose and 0.5 per cent NaCl. The medium was employed in small Erlenmeyer flasks in 50 cc. quantities. The technique was essentially the same as in the experiments with native proteins and with coagulated egg albumin. Bacterial counts were made by the plate method, immediately after inoculation and after definite intervals. Biuret and Sörensen determinations were also made. The results are given in table 10. They indicate that proteose resists direct bacterial attack, and thus falls in the same group with the native proteins and coagulated egg albumin.

B. prodigiosus, as in the experiments on native proteins, was able to develop in the test medium. This may be explained by

the ease with which the organism produces its proteolytic enzyme and by the unusual activity of this enzyme when present in very small amount. None of the other bacteria employed was able to increase in numbers in the proteose medium, all of the flasks remaining as clear as the control.

A series of tests was carried out also with the diphtheria bacillus, and the results recorded in table 11. Both strains of the organism used were unable to produce any change in the proteose content, as is shown clearly by the biuret tests, and corroborated by the Sörensen figures. Multiplication of the organisms was entirely at the expense of the meat extract. Whether any of the albumoses are affected by *B. diphtheriae* without losing their biuret-giving property remains undetermined. This

TABLE 11

Showing behavior of B. diphtheriae toward "purified" proteose

ORGANISMS	REACTION	SÖRENSEN TEST	BIURET TEST
B. diphtheriae no. 8, average 8 tests	14.0	28.0	1.0
B. diphtheriae (Y. M. S.), 9 tests	52.0	28.0	1.0
Control, uninoculated medium	32.0	28.0	1.0

Medium: 0.5 per cent proteose, 0.5 per cent beef extract, 0.5 per cent NaCl
Incubation at 30°C. for three weeks.

question is of particular interest in view of Hida's (1908) claim that deutero-albumose is essential in diphtheria toxin production.

THE UTILIZATION OF GELATIN AND CASEIN

Although a large number of organisms are known to liquefy gelatin, little has been done to determine whether the gelatin is actually utilized by the bacteria. Experimental evidence was sought in this work as to whether the power to liquefy gelatin carries with it ability to reduce it to its simple components. It has already been pointed out that the gelatinolytic and albumolytic properties of an organism may be distinct. The test medium employed in this investigation contained gelatin as the only known source of organic nitrogen.

The medium used in the first experiment contained 0.25 per cent commercial gelatin and 0.5 per cent NaCl. Liebig's meat extract (0.25 per cent) was added to half of the solution, and the two portions tubed separately in 10 cc. quantities. The tubes were inoculated with various organisms, and after an incubation period of three weeks the reaction and the Sörensen and biuret figures were determined. The results are not given in tabulated form, but may be found as such in the original thesis[3] at the Yale University Library. The reader is also referred to this thesis for other detailed statements and results which have not been incorporated in this paper.

B. subtilis, *B. ramosus* and *B. prodigiosus* were the only organisms studied which were able to completely utilize the gelatin, or to transform it into a-biuretic products. The proteus strains affected it only slightly, while *Staphylococcus aureus* and *B. cloacae* (liquefiers) made no impression whatever on the gelatin, thus showing the same behavior as *B. typhi*, *B. paratyphi*, *B. coli* and *B. Zenkeri*.

The apparent inability of *Proteus vulgaris* and *P. mirabilis* to decompose the gelatin was due to the limitations of the test fluid. Another test medium was prepared which contained opsine, a protein-free commercial product containing a large amount of readily available organic nitrogen for bacterial growth, besides the substances already cited. In this medium *Proteus vulgaris* and *P. mirabilis* were able to utilize from 50 to 70 per cent of the gelatin, as indicated by the biuret tests. The results with the other organisms were the same as in the gelatin solution which contained no opsine. The beef extract aided the metabolism of the proteus forms (not Zenkeri) to a small extent only.

It is difficult to offer a satisfactory explanation of the inability of *Staphylococcus aureus* and *Bacillus cloacae* to affect the decomposition of the gelatin beyond the gelatose stage. The evidence derived from both the Sörensen and biuret figures is sufficiently conclusive to show that the power of an organism to liquefy

[3] Doctorate thesis, Nathan Berman, May, 1917.

gelatin is not necessarily accompanied by the ability to decompose the gelatin and to seize upon it as food.

The digestion of casein has been a subject of study for many years. The observations reported by various investigators are to a large extent contradictory. This may be accounted for in part by a lack of uniformity in the methods. Blumenthal (1896) reported that *B. coli* was able to attack casein, and stated that proteolysis in milk is possible provided the acid formed from the lactose is neutralized as rapidly as it is formed. Taylor (1902) also claimed that *B. coli* could digest casein. He prepared a culture fluid containing purified casein. The inoculated medium was examined after a definite interval for amino acids. Being unable to detect any amino acids, he assumed that the casein was decomposed into proteoses and peptones. From cultures of *P. vulgaris* he was successful in isolating several amino acids, however.

Barthel (1915) studied the digestion of casein by milk-souring bacteria. Rosenow (1913) reported that he was able to obtain an enzyme from a pneumococcus broth culture which could decompose blood serum, but not casein.

In the present investigation the behavior of certan organisms was studied in a medium containing purified casein. The medium contained the following ingredients: 0.125 per cent casein, 0.5 per cent Liebig's meat extract and 0.5 per cent NaCl. The solution was tubed in 10 cc. quantities. A test for unchanged casein was always made with dilute acid. Chief reliance was placed on the biuret and the Sörensen tests.

The behavior of the organisms was the same in this medium as in those containing purified proteose and gelatin. The subtilis members, *B. prodigiosus* and the proteus forms (vulgaris and mirabilis) were able to break down the casein. None of the other organisms studied (*Staphylococcus aureus* and *albus*, *B. typhi A* and *B. B. coli* and *B. diphtheriae*) possessed the ability to affect the casein in the slightest degree, even during three weeks of incubation at 30°C. That *B. coli* and its close allies cannot decompose casein should not be surprising, though the facts as established here are contrary to the observations

of Taylor (1902). It is not at all improbable that what Blumenthal reported as digestion was in reality only a solution of the casein in an alkaline medium.

THE UTILIZATION OF THE PRODUCTS OF WITTE'S PEPTONE AND OF CASEIN OBTAINED BY PARTIAL DIGESTION WITH TRYPSIN

The inability of many organisms to develop in the common laboratory media is due to the absence in these media of sufficient food material which is immediately available for cell nutrition. That a large part of Witte's peptone, as well as gelatin, casein, unchanged native proteins and coagulated egg albumin, is valueless as a source of organic nitrogen, in so far as the large group of gelatin-non-liquefying organisms at least is concerned, has been demonstrated in this investigation. Smith (1897) pronounced plain peptone solution to be a poor culture fluid. He stated that some organisms failed to grow in Witte's peptone. Rivas (1912) found that short tryptic digestion of peptone enhanced its value as a culture medium. Hottinger (1913) who denounced Witte's peptone as unsatisfactory for culture purposes suggested a pancreatic digestion product of meat as a substitute.

Dalimier and Lancereaux (1913) found the protein-free product, "opsine," which is rich in amino acids and other simple nitrogenous substances, to serve as a good culture medium for all of the many organisms studied, including some very fastidious pathogens. These results, with a single exception, were recently corroborated by Robinson and Rettger. Cole and Onslow (1916) suggested a pancreatic digestion product of casein as a good medium, and Distaso (1916) succeeded in obtaining good growths of pathogenic organisms in a solution which contained as its only source of nitrogen serum which had been digested with trypsin.

In the present work solutions of Witte's peptone and of casein were digested with commercial trypsin. The digestion was allowed to continue for five hours at 45°C., after which the liquid was immediately sterilized, and 0.25 per cent beef extract

and 0.5 per cent NaCl added. The media were tubed in 10 cc. quantities, and inoculated with the same organisms used in the preceding tests. The methods of determining the utilization of biuret-giving substances were also the same as those employed throughout the earlier work.

The increased utilization of the predigested peptone was plainly evident. The action of the trypsin almost doubled the amount of nitrogen available for the non-proteolytic organisms. In several instances 65 per cent of the biuret-giving substances of the peptone digestion product was utilized by this group of bacteria, and 35 per cent of the biuret-positive casein digestion product. Aside from the great reduction in the biuret figures, and the high Sörensen titration, the nutrient property of the medium was evidenced by the luxuriance of bacterial growths. It is to be assumed that the large reduction by the gelatin-non-liquefiers in the biuret figure was due to the preponderance of polypeptids of comparatively simple constitution.

GENERAL DISCUSSION AND CONCLUSIONS

The results obtained in the present investigation confirm those of previous experiments, and further justify the assumption that bacteria require food substances which are of simple constitution and whose properties are such that they readily admit of absorption by the bacterial cell. This view is in harmony with that of Loewi (1902), Abderhalden, etc. as applied to animal nutrition. It furnishes an explanation of the inefficiency of the ordinary laboratory culture media, and particularly solutions of Witte's peptone, for rapid and abundant production of bacterial growth.

Commercial peptones are highly complex products, containing, among other things, different albumoses and so-called peptones, amino acids, particularly the primary, and other simple nitrogen-containing substances. No two brands of commercial peptone are of the same composition, nor is it likely that any one product is uniform. Numerous tests have shown that the amino acid content of some products is decidedly

greater than that of others, while the proportion of the albumose and complex polypeptid fraction is correspondingly less.

The presence of a peptolytic enzyme in cultures of coli, typhi and other gelatin-non-liquefying bacteria has apparently been demonstrated. This erepsin-like enzyme is, however, decidedly weak, and further differs from erepsin of the animal body in that it does not attack casein. The decomposition of biuret-giving material in commercial peptone is, without doubt, limited to the substances of relatively simple constitution, or the lower polypeptids. This has been demonstrated by the inability of the non-proteolytic organisms in question to attack "purified" proteose, and in a measure by the peculiar shading off of the biuret color, during prolonged periods of incubation, from the characteristic light purple color obtained with a solution of peptone to the deep purple or violet which is given by proteose and albumin solutions with the alkaline copper sulphate.

It may of course be questioned whether the decomposition of the simple polypeptids of the "peptone fraction" requires any enzyme action, and whether these substances are not sufficiently simple and soluble to be absorbed and utilized directly by the bacterial cell. Such an explanation of the availability of the simple amino acids for bacterial nutrition is quite plausible, since they are very soluble and dialyzable; and further, it would appear improbable that specific enzymes for the many different amino acids are elaborated.

Even the gelatin-liquefying and proteolytic bacteria are slow to make use of the proteoses and peptone in commercial "peptone." Their initial development is at the expense of the simpler nitrogenous compounds, and a reduction in the biuret-giving substances occurs only after a period of preliminary cultural development, this period often extending over at least a day or two. In this preparatory interval the proteolytic enzyme is formed which is necessary in the utilization of the soluble protein material. That the production of the enzyme which attacks gelatin is as a rule a slow process is well shown in the ordinary gelatin liquefaction tests.

The ability to liquefy gelatin is not in itself a proteolytic or even a gelatinolytic function, for some organisms which can liquefy gelatin are unable to carry the change beyond the gelatose stage, and fail to decompose "purified" proteose and casein.

Members of the coli-typhi-paratyphi group of organisms studied are without ability to decompose gelatin and casein. They are unable also to affect the "proteoses" in commercial peptone, and in their reduction of the biuret-giving substances attack and decompose only the simpler of these components, presumably the lower polypeptids; the action on even these relatively simple substances is slow and delayed. *Bacillus cloacae* is not here included in the above group of organisms, though its action in the media containing the above substances is almost, if not entirely, the same as that of *B. coli*, etc.

The value of a cultural medium for bacteria depends on the immediately available food substances which it contains. For this reason it appears quite reasonable that those commercial peptones which contain the largest amount of amino acids and other nitrogenous products of simple composition are most conducive to active and abundant bacterial development, every thing else being equal. This supposition is fully borne out by experiment. The question as to whether such peptones are the most satisfactory for toxin production in a medium requires further and extensive investigation.

The conclusions may be summarized briefly as follows:

1. Bacteria are unable to decompose coagulated native protein when there is no other source of available nitrogen in the test medium.

2. There is reason to believe that purified proteose also is resistant to direct attack by bacteria.

3. Gelatin-non-liquefying bacteria and some of the liquefiers are feeble in their action on Witte's peptone. A much better utilization of the biuret-giving substances occurs in media containing commercial peptone which has been subjected to more extensive digestion in the process of preparation, as for example certain of the American brands.

4. The ability of an organism to liquefy gelatin is no sure indication of its proteolytic properties.

5. Gelatin and casein resemble proteose in their resistance to bacterial attack.

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THE INFLUENCE OF CARBOHYDRATE ON THE NITROGEN METABOLISM OF BACTERIA

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The use of fermentable sugars, alcohols and glucosides in bacteriological culture media has marked a distinct advance in the present methods of studying and classifying bacteria. Chief emphasis has been placed, however, on the question as to whether certain given organisms attack these substances with resultant acid and gas production. Among those who have given their attention to the utilization of carbohydrates and their influence on nitrogen metabolism considerable difference of opinion exists; and a review of the literature shows that further and more definite information on this subject is indeed desirable.

Liborius (1886) observed that the presence of sugar did not prevent liquefaction of gelatin by *Bacillus subtilis*, *Pseudomonas fluorescens* and *Bacillus pyocyaneus*. Winternitz (1892), as the result of animal feeding experiments, came to the conclusion that glucose and lactose inhibit the bacterial decomposition of protein. Glenn (1911) claimed that in a medium containing sugar and protein material the carbohydrate is first attacked, and that the protein is subsequently decomposed, providing the acidity produced in the complete fermentation of the sugar is not too great. According to Glenn, therefore, no protein utilization takes place in the presence of fermentable carbohydrate.

Kendall, Day and Walker (1913) emphasized the preference of bacteria for sugars. According to them nitrogen metabolism in carbohydrate media is at its minimum. Aubel and Colin (1913) observed that fermentable sugars inhibit color production of bacteria. A year later these same authors found that the addition of glucose to a medium lessens or inhibits the pro-

duction of ammonia. Kendall, Day and Walker (1915) failed to demonstrate the formation of an active proteolytic enzyme by *Proteus vulgaris* in either glucose broth or glucose gelatin. The enzyme produced in carbohydrate-free medium, and freed from the bacteria by filtration, liquefied glucose gelatin as readily as plain gelatin, however. According to these workers, the presence of sugar delays the formation of the proteolytic enzyme until the carbohydrate has been used up. Jones (1916) also concluded that the failure of *P. vulgaris* to decompose the protein in a glucose gelatin medium is due to the absence of enzyme formation. He states that acidity plays no part in influencing the behavior of the organism. Sears (1916) concluded that glucose had a sparing action on nitrogen metabolism. Kligler (1916) offers a similar explanation, and states that the proteolytic enzyme of *Proteus vulgaris* is not formed in the early stages of carbohydrate metabolism.

Other investigators observed the influence of sugar on indol production. Baginski (1889) reported that he was unable to obtain an indol test in the presence of lactose. Péré (1892) concluded that a positive test for indol indicated a complete disappearance of sugar. He affirms that carbohydrates are more available than protein, and are utilized first. Distaso (1913) showed that indol formation may occur in a carbohydrate medium containing free tryptophan. *B. coli* was employed as the test organism. According to this writer the more available substances in a medium are used first, and as a consequence indol is not formed in ordinary sugar media. De Graaff (1909) had previously observed the inhibiting effect of glucose on indol production.

Rougentzoff (1913) claimed that the carbohydrates, glucose, fructose and lactose, and the alcohol mannite, which are fermented rapidly by *Bacillus coli*, inhibit indol production. Indol was formed, however, in media containing maltose, sucrose and dulcite. The titratable acidity was taken as an index of the rapidity with which different saccharides were decomposed. Fischer (1915) reported somewhat different results. He observed that glucose alone inhibited indol formation, while lactose, maltose,

galactose and fructose were without effect. He believed that the acid formed played no rôle, since the addition of calcium carbonate to the medium did not influence the results. Fischer concluded that the inhibitive action of the glucose is due to an inactivation of the proteolytic enzyme of the organism. This view is partly in harmony with the theory recently advanced by Homer (1916), according to which a chemical union is formed between the aldehyde group of the glucose and a portion of the tryptophan molecule. The combination is more resistant to attack than free tryptophan.

EXPERIMENTAL

A study of the behavior of certain organisms in glucose peptone broth was first undertaken. The test medium contained peptone (Witte) 0.25 per cent, sodium chloride 0.5 per cent, and glucose 1.0 per cent. The same medium minus the sugar was used for control purposes. The test and control solutions were transferred in 10 cc. quantities to test tubes, and inoculated with the test organisms. At the end of four weeks of incubation at 37°C. the water of evaporation was replaced and biuret tests made according to the method of Vernon (1904). The Sörensen (1908) method for the determination of amino acids was also carried out, as well as the Benedict test for the presence of unchanged sugar. The results are presented in table 1.

Barring one exception, no protein decomposition occurred in the presence of glucose. *Bacillus subtilis* alone was able to digest the peptone in the glucose-containing medium. In every other instance the biuret and Sörensen tests gave the same figures as in the uninoculated controls. The acidity in the glucose tubes was increased, with the exception of the case of *Bacillus ramosus*. Owing to the unfavorable temperature for this organism, *Bacillus ramosus* grew but sparingly. The digestion of the peptone by *Bacillus subtilis*, even though the glucose was not completely fermented, is an indication that a glucose-utilizing organism may secrete a proteolytic enzyme in the presence of a fermentable sugar.

TABLE 1

Showing the effect of glucose on nitrogen metabolism

ORGANISM	REACTION		SÖRENSEN		BIURET		SUGAR	
	C	D	C	D	C	D	C	D
Control uninoculated	4.0	4.0	4.0	4.0	1.0	1 0	–	+
B. subtilis	2.0	6.0	22.0	28.0	0.0	0.0	–	+
B. ramosus	6.0	4.0	14.0	8.0	0.75	1.0	–	+
P. vulgaris	4.0	12.0	8.0	6.0	0.0	1.0	–	+
P. mirabilis	2.0	14.0	8.0	6.0	0.0	1.0	–	+
Staph. aureus	2 0	10.0	4.0	6.0	0.7	1.0	–	+
Staph. aureus, II	2.0	10.0	8.0	6.0	0.7	1.0	–	+
Staph. albus	4.0	10.0	16.0	4.0	0.7	1.0	–	+
B. typhi	0.0	14.0	6.0	4.0	0.8	1.0	–	+
B. paratyphi A	2 0	14.0	6.0	6.0	0.8	1.0	–	+
B. coli	2.0	20.0	4.0	4.0	0.85	1.0	–	+
B. diphtheriae	4.0	6.0	6.0	6.0	0.85	1.0	–	+

The composition of Media C and D is as follows:

Medium C	*per cent*	*Medium D*	*per cent*
Peptone	0.5	Peptone	0.5
NaCl	0.25	NaCl	0.25
		Glucose	1.0

A more extensive study of the influence of carbohydrates on the protein metabolism of *Bacillus subtilis* was then conducted. Five different media were prepared as follows:

MEDIUM 1	MEDIUM 2	MEDIUM 3	MEDIUM 4	MEDIUM 5
Peptone, 0.5 per cent Beef extract, 0.25 per cent NaCl, 0.5 per cent	1 plus 0.2 per cent glucose	1 plus 0.5 per cent glucose	1 plus 0.2 per cent lactose	1 plus 0.5 per cent lactose

Medium 1 was used as control. The five solutions were transferred to test tubes (10 cc.), each tube but the control being inoculated with a pure strain of *Bacillus subtilis*. A series of fifteen tubes was used for each medium. Tubes from each series were taken from the incubator on alternate days, and tested

as in the preceding experiment. The incubation periods varied from one to twenty-seven days, a constant temperature of 30°C. being employed. The results are not given here in tabulated form,[1] but may be summed up briefly as follows.

The titratable acidity was at no time high. Towards the end of the experiment the reaction in the glucose and lactose tubes was practically the same as that of the uninoculated controls, 10, as compared with 28 and 30, the highest points of acidity. The Sörensen titration increased simultaneously in all of the inoculated media, and in practically the same degree. The biuret tests indicate the same metabolic changes as the Sörensen titration. The peptone was completely utilized in each of the five media before three weeks of incubation had elapsed. The slow rate at which the sugars were fermented is noteworthy. The first evidence of disappearance of the sugars, even in the tubes originally containing only 0.2 per cent of the carbohydrates, occurred after two weeks, while twenty-five days were required for their complete disappearance from the tubes originally containing 0.5 per cent.

A similar study was made with *Bacillus coli* and *Proteus vulgaris*. The details of the experiment were the same as in the previous experiment, except that with these two organisms two additional tests were conducted, namely the indol and methyl red tests. The Salkowski method of determining the presence of indol was employed. The test was made by adding 0.25 cc. of concentrated sulphuric acid to a few cubic centimeters of the fluid under observation, immediately cooling the mixture in cold water, and slowly adding a 0.5 per cent solution of sodium nitrite while the tube was held in an almost horizontal position. This method permits of a minimum amount of diffusion, so that the color is made all the more apparent in the case of a positive test. Difficulty in procuring the Ehrlich aldehyde prevented the use of this agent until near the close of the investigation. A comparative study of the two methods has shown them to be equally reliable in our own hands.

[1] The original tables may be consulted in the doctorate thesis (Berman), a copy of which is in the possession of each of the writers and of the Yale University Graduate School.

The methyl red test of Clark (1915) was employed because it gives an index of the 'true reaction' of the test media. This test has proven itself to be of much value.

The behavior of *Bacillus coli* in the media containing the glucose and lactose is of particular interest. The biuret and Sörensen tests gave essentially the same figures for the control medium (plain peptone broth) as in previous experiments. The indol appeared on the third day, and remained throughout the course of the experiment. The final reaction was practically neutral to phenolphthalein. In the media containing glucose the results were quite different. The hydrogen ion concentration was markedly increased, both glucose media (0.2 per cent and 0.4 per cent) becoming methyl red positive. In the tubes containing 0.2 per cent of glucose the sugar was completely fermented by the third day. Little metabolism seemed to take place after this. The titratable acidity remained high. The Sörensen values were almost the same as for the controls. No indol tests were obtained in the glucose tubes even after four weeks. Although 0.2 per cent glucose was used up in three days, 0.5 per cent was not completely fermented in a period of four weeks. The biuret tests indicated that no "peptone" digestion had taken place in the sugar-containing tubes.

The behavior of *Bacillus coli* in the lactose media was about the same as in the glucose broth. In the tubes containing 0.2 per cent lactose the methyl red test suddenly changed from positive to negative. The indol test became positive with the decrease in hydrogen ion concentration. It appeared as if conditions gradually became favorable for growth, and that the rate of bacterial development increased as the acidity was neutralized. The coördination of the different tests is of special significance.

Fermentable sugar exerts the same influence on the metabolism of *Bacillus vulgaris* as on that of *Bacillus coli*. In the medium containing no carbohydrate the protein is completely broken down by the former organism, however. Within twenty-four hours there was appreciable indol formation. Indol was not produced, on the other hand, in any of the glucose-bouillon

tubes. The hydrogen ion concentration in these reached a point which inhibited the metabolic activities of the organism. This was apparent from the constancy of the biuret and the Sörensen figures. *Proteus vulgaris*, like *Bacillus coli*, was unable to ferment 0.4 per cent glucose completely, although 0.2 per cent was utilized in twenty-four hours.

Lactose was without effect on the protein metabolism of *Proteus vulgaris*. This was to be expected, of course, as this organism is generally regarded as being unable to attack lactose, in spite of a few claims to the contrary. The acidity, biuret and Sörensen figures, and the indol and sugar tests were the same in the lactose broth as in the plain peptone bouillon.

The above observations strongly indicate that the H ion concentration plays the important rôle in the inhibition of nitrogen metabolism in a medium containing a fermentable sugar. The failure of certain organisms to attack protein in the presence of carbohydrates is firmly established. That this failure is dependent on a coincident rise in the acidity of the medium is fully supported by the following experiments.

Media of the same composition as those already described were employed, with and without the addition of dipotassium phosphate. The media were made up as follows:

B	*2D*	*2DP*
Peptone, 0.5 per cent Beef extract, 0.25 per cent NaCl, 0.5 per cent	1 + 0.2 per cent glucose	2D plus 0.5 per cent K_2HPO_4
4D	*4DP*	*2L*
B plus 0.4 per cent glucose	4D plus 0.8 per cent K_2HPO_4	B plus 0.2 per cent lactose
2LP	*4L*	*4LP*
2L plus 0.5 per cent K_2PO_4	B plus 0.4 per cent lactose	4L plus 0.8 per cent K_2HPO_4

The dipotassium phosphate was used to serve as a buffer. The organisms employed were *Bacillus coli* and *Proteus vulgaris*,

both glucose and lactose media being inoculated with the colon bacillus, while the experiments with *Proteus vulgarus* were carried out in lactose broth alone, aside from the plain peptone broth cultures. Only the results showing the behavior of *Proteus vulgaris* in the glucose media are presented here in tabulated form (table 2), as they are thoroughly representative and well illustrate the influence of buffer action of the phosphate on the decomposition of the peptone in the presence of a fermentable sugar. With one exception, the results differ little from those obtained with *Bacillus coli* in both the glucose and lactose media, aside from the limited "peptone" utilization by *Bacillus coli* under even the most favorable conditions for protein metabolism.

The striking influence of the buffer reagent, dipotassium phosphate, on protein metabolism is most marked. The acidity of all of the sugar media increased during the first twenty-four hours. Subsequently the titratable acidity rapidly diminished in the solutions containing the phosphate, while in the other tubes it remained fairly constant. The biuret figures were in harmony with the Sörensen data. The reduction in the amounts of biuret-giving substances in the sugar media containing the phosphate was essentially the same as has been observed in plain peptone broth, the protein completely disappearing in two to three weeks. On the other hand, no reduction was noted in the sugar media to which no di-basic phosphate had been added. The Sörensen value of the phosphate tubes was increased two to three fold, while in the glucose broth, and in the case of *Bacillus coli* in lactose broth, containing no added phosphate, it remained practically constant.

The indol tests also indicated that protein metabolism occurred only in the solutions containing the buffer. In no instance was a positive indol test obtained in the glucose peptone broth to which no phosphate had been added. In both of the 0.2 per cent glucose media (with and without the buffer) *Bacillus coli* completely fermented the sugar, within twenty-four hours, while 0.4 per cent glucose was used up entirely only in the medium which contained the phosphate, from which it disappeared within the first twenty-four hours.

TABLE 2

Showing the influence of a buffer (K_2HPO_4) *on the protein metabolism of Proteus vulgaris in a glucose medium*

	REACTION				SÖRENSEN TEST				BIURET TEST				INDOL				SUGAR				METHYL RED			
	2D	2DP	4D	4DP	2D	2DP	4D	4DP	2D	2DP	4D	4DP	2D	2DP	4D	4DP	2D	2DP	4D	4DP	2D	2DP	4D	4DP
Control.....	12.0	22.0	12.0	22.0	14.0	14.0	14.0	14.0	1.0	1.0	1.0	1.0	−	−	−	−	+	−	+	+	−	−	−	−
Inoculated																								
1 day..	28.0	36.0	30.0	34.0	16.0	14.0	16.0	10.0	1.0	1.0	1.0	1.0	−	+	−	−	+	+	+	+	+	−	+	−
3 days.	30.0	24.0	34.0	38.0	16.0	20.0	16.0	12.0	1.0	0.9	1.0	0.8	−	+	−	+	−	+	+	+	+	−	+	−
5 days.	30.0	28.0	30.0	34.0	16.0	24.0	14.0	18.0	1.0	0.9	1.0	0.9	−	+	−	+	−	+	+	+	+	−	+	−
7 days.	30.0	6.0	28.0	10.0	16.0	40.0	16.0	32.0	1.0	0.7	1.0	0.7	−	+	−	+	−	+	+	+	+	−	+	−
9 days	30.0	0.0	30.0	6.0	16.0	40.0	16.0	36.0	1.0	0.6	1.0	0.6	−	+	−	+	−	+	+	+	+	−	+	−
11 days.	28.0	*2.0*	30.0	*2.0*	18.0	42.0	16.0	42.0	1.0	0.5	1.0	0.35	−	+	−	+	−	+	+	+	+	−	+	−
13 days.	28.0	*2.0*	30.0	*2.0*	16.0	40.0	16.0	42.0	1.0	0.5	1.0	0.3	−	+	−	+	−	+	+	+	+	−	+	−
15 days.	30.0	8.0	26.0	*2.0*	16.0	22.0	16.0	42.0	1.0	0.7	1.0	0.15	−	+	−	+	−	+	+	+	+	−	+	−
17 days.	28.0	*4.0*	30.0	*4.0*	16.0	46.0	16.0	46.0	1.0	0.3	1.0	0.2	−	+	−	+	−	+	+	+	+	−	+	−
19 days.	28.0	*2.0*	30.0	*2.0*	18.0	44.0	18.0	46.0	1.0	0.2	1.0	0.0	−	+	−	+	−	+	+	+	+	−	+	−
21 days.	30.0	*4.0*	32.0	*2.0*	16.0	40.0	16.0	42.0	1.0	0.0	1.0	0 0	−	+	−	+	−	+	+	+	+	−	+	−
23 days.	28.0	*4.0*	32.0	*6.0*	16.0	40.0	16.0	44.0	1.0	0.0	1.0	0.0	−	+	−	+	−	+	+	+	+	−	+	−
25 days.	28.0	*2.0*	32.0	*4.0*	16.0	40.0	14.0	42.0	1.0	0.0	1.0	0.0	−	+	−	+	−	+	+	+	+	−	+	−
27 days.	30.0	8.0	32.0	6 0	16.0	32.0	18.0	30.0	0.95	0.0	1.0	0.0	−	+	−	+	−	+	+	+	+	−	+	−

Italics indicate alkalin reaction to phenolphthalein.

Medium 2D
Peptone, 0.5 per cent
Beef extract, 0.25 per cent
NaCl, 0.5 per cent
Glucose, 0.2 per cent

Medium 2DP
Peptone, 0.5 per cent
Beef extract, 0.25 per cent
NaCl, 0.5 per cent
Glucose, 0.2 per cent
K_2HPO_4, 0.5 per cent

Medium 4D
Peptone, 0.5 per cent
Beef extract, 0.25 per cent
NaCl, 0.5 per cent
Glucose, 0.4 per cent

Medium 4DP
Peptone, 0.5 per cent
Beef extract, 0.25 per cent
NaCl, 0.5 per cent
Glucose, 0.4 per cent
K_2HPO_4, 0.8 per cent

Proteus vulgaris manifested a peculiar behavior in the glucose tubes containing the di-basic phosphate, in which it failed to utilize the carbohydrate completely (see table 2). Even 0.2 per cent glucose was not completely fermented in the presence of the buffer. These results were indeed unexpected, and the tests were repeated, with identical results. It appears from these data that *Proteus vulgaris* readily attacks the nitrogen-containing substances in the absence of excessive acidity, and in preference to the residuary carbohydrate.

In all of the buffered media the methyl red test was negative, while in the sugar-containing solutions the hydrogen ion concentration was sufficiently increased within twenty-four hours to give the color reaction with methyl red.

GENERAL DISCUSSION

The influence of fermentable carbohydrates on nitrogen metabolism of bacteria is clearly shown in the foregoing experiments. The failure of certain organisms to attack protein or other complex nitrogenous foods in a medium containing carbohydrate is due to the accumulation of products which inhibit growth. The nitrogen which is required in the cell reproduction of the bacteria in the sugar medium is supplied by relatively simple substances, as for example amino acids and purin bases, which are always present in the ordinary media containing commercial peptone or meat extract, although in small amount.

The information furnished by the titratable acidity figures is of little value. Reactions which would ordinarily be considered too high apparently had no inhibitive action on continued bacterial development. The futility of adjusting the reaction to phenolphthalein was pointed out by Clark (1915) and further emphasized by Burton and Rettger (1917). It is now well known that culture fluids giving the same titration values may differ markedly in their hydrogen ion concentration. As was shown in the present work, a culture giving a methyl red negative test may have a titratable acidity twice as great as one that is methyl red positive.

The ability of *Bacillus subtilis* to break down protein in the presence of fermentable sugar, and in the absence of an added buffer, may be explained as follows. This organism attacks glucose slowly, and for this reason it is able to produce its proteolytic enzyme before the hydrogen ion concentration reaches a point unfavorable to further growth. When the enzyme is thus formed the products of the nitrogen metabolism neutralize the acid, at least in a measure, and the metabolism therefore continues uninterruptedly.

It is probable that the metabolism of *Bacillus coli* in a glucose or lactose medium in which much of the nitrogen is in a form directly available for utilization by the organism is such as to maintain a hydrogen ion concentration suitable for normal development without the aid of a buffer. In a case like this the nitrogen metabolism is rapid enough to neutralize the acid formed from the sugar decomposition. Such a condition has been shown in cultures of *Bacillus coli* in lactose media to which tryptophan was added (Distaso, 1913). Indol production could be demonstrated easily.

A recent experiment in which the metabolism of *Bacillus cloacae* was investigated clearly indicates that this organism behaves as *Bacills subtilis* does, except that the sugar is fermented more rapidly by the former. The final products of glucose and lactose fermentation by *Bacillus cloacae* are largely carbon dioxide and hydrogen, and hence the hydrogen ion concentration is not altered greatly.

The four organisms, *B. subtilis*, *B. cloacae*, *B. coli* and *P. vulgaris*, illustrate three distinct types of metabolism. *Bacillus subtilis* attacks protein in the presence of fermentable sugar, because the sugar is fermented slowly and the nitrogen and carbohydrate metabolism are maintained in equilibrium. *Bacillus cloacae* is also able to regulate its own environment, although the sugar is fermented very rapidly, but comparatively little acid occurs among the end products, and hence bacterial development and a nitrogen metabolism are not inhibited. *Bacillus coli* and *Proteus vulgaris* rapidly ferment glucose with the formation of large amounts of acid. Providing there is sufficient

sugar present, these organisms may produce enough acid in twenty-four hours to prevent further metabolism. The presence of a buffer, like dipotassium phosphate, tends to prevent such inhibition.

Kendall and Walker's conception that the presence of glucose delays the production of the proteolytic enzyme can not be accepted. A bacterial proteolytic enzyme, as a rule, is not produced within the first twenty-four hours, but requires a longer period before it makes its appearance in detectable quantities. Previous to enzyme formation some anabolism must take place, unless some enzyme has been transferred in the process of inoculation. In the tests in which the buffer reagent was employed the proteolytic enzyme appeared as soon in the sugar media as in the plain bouillon.

A positive indol test does not necessarily indicate that the sugar has been entirely fermented. Homer's explanation that the presence of glucose in a medium prevents indol formation because it forms a chemical combination with the tryptophan is therefore based on a false assumption.

The present investigation has shown conclusively that fermentable sugars in moderate amounts do not affect the nitrogen metabolism of bacteria, providing experimental conditions are favorably maintained, or in other words, under conditions of favorable environment. The common belief in a so-called "sparing action" of sugars in a protein medium is untenable, in the light of these experiments. According to this idea protein nitrogen is left unattacked, and thus spared from all participation in the metabolism. There is a true sparing action, however, in the sense that the nitrogen is utilized merely for growth, and that the sugar furnishes the energy. For example, the more luxuriant growth of the organisms here used in the glucose media containing the buffer as compared with plain peptone broth, may be explained on the basis that the nitrogen was used for growth, and that the sugar furnished the necessary energy supply. In plain peptone broth the nitrogenous food material furnishes both the energy and the nitrogen for necessary cell metabolism.

CONCLUSIONS

1. The presence of a carbohydrate in a culture medium may inhibit protein metabolism, depending on the nature of the medium and on the type of the organism.

2. The presence of sufficient buffer in a medium encourages continued normal nitrogen metabolism.

3. A utilizable carbohydrate in a medium has a true 'protein sparing' effect providing the hydrogen ion concentration is maintained within suitable limits.

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STUDIES IN THE CLASSIFICATION AND NOMENCLATURE OF THE BACTERIA

VIII. THE SUBGROUPS AND GENERA OF THE ACTINOMYCETALES

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Order III. **Actinomycetales.** *Nom. Nov.*

Synonyms:

Actinomycetes Balbiani, 1886, p. 542.

Trichobacteriacei Fischer, 1895, p. 138 in part.

Mold-like organisms, not typically water forms, saprophytic or parasitic. Sheath not impregnated with iron, true hyphae with branching often evident, conidia may be developed, but never endospores. Without granules of free sulphur and without bacteriopurpurin. Never producing a pseudoplasmodium. Always nonmotile.

The order *Actinomycetales* contains a single family, *Actinomycetaceae.*

Family I. **Actinomycetaceae.** Fam. nov.

Characters same as those of the order.

The following names have been used for genera which may be included in this family.

Actinobacillus Brumpt, 1900, p. 849
Actinobacterium Haass, 1906, p. 180
Actinocladothrix Affanassieff, 1888, p. 79
Actinomyces Harz, 1877, p. 125
Cohnistreptothrix Pinoy, 1911
Viscomyces Rivolta and Micellone, 1879, p. 145
Leptotrichia Trevisan, 1879, p. 138

Micromyces Gruber, 1891, p. 648
not *Micromyces* Dangeard, 1888, p. 55
Nocardia Trevisan, 1889, p. 9
Streptothrix Cohn, 1875, p. 186
not Corda, 1839
Thermoactinomyces Tsilinsky, 1899, p. 500
Rasmussenia De Toni and Trevisan, 1889, p. 930

The genus *Actinobacterium* has had no definite specific names ascribed to it.

The following are invalid because previously used for other distinct groups of organisms: *Micromyces* and *Streptothrix*.

The name *Actinocladothrix* was used by Affanassieff in the combination *Bacterium actinocladothrix*, but several authors have listed the name as though it had been used as a genus.

The following generic names are therefore to be considered, or at least are not invalid for any of the preceding reason: *Actinobacillus*, *Actinomyces*, *Cohnistreptothrix*, *Discomyces*, *Leptotrichia*, *Nocardia*, *Thermoactinomyces*, *Rasmussenia*.

The genera may be differentiated by the following key.

Key to the genera of Actinomycetaceae

A. No evident *aerial* threads or conidia formed. Usually parasitic. Often anaërobic or microaërophilic.
1. Threads usually not branched.
a. Threads disjointing very readily; long mycelial threads uncommon. Genus 1. *Actinobacillus*
b. Threads longer, not disjointing into short rods. Genus 2. *Leptotrichia*
2. Threads more or less branched, frequently clubbed in tissues. Genus 3. *Actinomyces*
B. Aërial threads and conidia evident on culture media.....Genus 4. *Nocardia*

Genus 1. **Actinobacillus** Brumpt, 1900, p. 849

Filament formation; resembling streptobacilli. In lesions no mycelium formed, but at peripheries finger shaped branched cells are visible. Gram negative. Not acid fast.

Possibly the genus belongs with the *Bacteriaceae*. It is evidently a transition form.

The type species is *Actinobacillus lignieresi* Brumpt, the cause of actinobacillosis in cattle.

Genus 2. **Leptotrichia** Trevisan, 1879, p. 138

Synonyms:
Leptothrix Robin, 1847, p. 345
not *Leptothrix* Kuetzing, 1843, p. 198
Rasmussenia Trevisan, 1889, p. 930

Rod shaped or filamentous cells, non motile, unbranched, without aërial hyphae or conidia; parasites or facultative parasites.

The type species is *Leptotrichia buccalis* (Robin) Trevisan. This genus is commonly termed *Leptothrix*, but certainly forms as unlike as *Leptothrix ochracea* and *Leptotrichia buccalis* do not belong in the same genus. *Leptotrichia* was created by Trevisan in 1879 with *L. buccalis* as the only species, but in 1889 he enlarged the genus, removing the mouth forms to the genus, *Rasmussenia*.

Genus 3. **Actinomyces** Harz, 1877, p. 125

Synonyms:
Streptothrix Cohn, 1875, p. 186
not *Streptothrix* Corda
Discomyces Rivolta and Micellone, 1879, p. 145
Micromyces Gruber, 1891, p. 648
not *Micromyces* Dangeard, 1888, p. 55
Nocardia Trevisan (in part)
Oospora Sauvageau and Radais, 1892, p. 242
not *Oöspora* Wallroth, 1833, p. 182
Cohnistreptothrix Pinoy, 1911

Branched filaments, resembling mycelium, breaking up into segments which may function as conidia. Usually parasitic. Clubbed ends conspicuous in lesions. Not producing aërial hyphae or conidia.

The type species is *Actinomyces bovis* Harz, the cause of bovine actinomycosis.

Genus 4. **Nocardia** Trevisan, 1889, p. 9

Synonyms:

Actinomyces of many authors
Streptothrix of many authors
Thermoactinomyces Tsilinsky, 1899, p. 500

Branched filaments, resembling a mycelium, readily breaking up into segments. Usually saprophytic. Aërial threads and conidia commonly produced.

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THE CLASSIFICATION OF THE ACIDURIC BACTERIA

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The proliferation of the aciduric bacteria apparently depends upon the almost exclusive utilization of carbohydrates or carbohydrate-like substances. The corollary of this property, that is, the ability to survive in the presence of considerable amounts of acid is made use of in their isolation. The titles applied to this group have varied with the authors who have studied it. The term "Lactic acid bacteria" is certainly too broad, since its use would force the inclusion of organisms that are of an entirely different type, such as *B. coli*. "Bulgaricus" and "Caucasicum" are unsuitable terms because these bacilli are neither the most frequently occurring nor the typical members of this group. "Acidophilus," while more nearly appropriate, is still inexact, since this bacillus is characterized by its acid resisting rather than by its acid "loving" properties. Upon the whole, the word "aciduric," adopted by Kendall (1910) appears to be the most fitting term under which to group these organisms.[1]

The distribution of the aciduric bacteria calls for no comment, their ubiquity as a group has long been known. The distribution of individual members, however, is less well understood. In 1909 Heinemann and Hefferan published a paper in which it was stated that *B. acidophilus* and *B. bulgaricus* were identical. It seemed to the writer that much of the work on this subject, both preceding and following that of these authors, was not as convincing as might be, owing to the failure of practically all

[1] Distaso (Cent. f. Bakt. Orig. 1911, **59**, 48) employed the term acid-tolerant.

workers to provide a suitable medium for the cultivation of these bacteria.

In 1914 Rahe was able to show that these organisms would develop with a very satisfactory degree of luxuriance in unneutralized meat-peptone-broth containing a suitable carbohydrate. We have carried various strains of aciduric organisms for years in this broth. Although the writer has in subsequent communications tried to place sufficient emphasis on this fact, the fallacy still persists that these bacteria are not readily grown on the "usual" laboratory media.

Once the difficulty attending the cultivation of the aciduric bacteria was removed, an examination of the cultural characteristics of a number of strains brought to light several significant differences between the members of this group. It became evident that the Bulgarian bacillus could not utilize maltose (some strains could not ferment sucrose) and in this respect differed from the great majority of aciduric bacilli found in the human intestine and elsewhere. The latter could be conveniently grouped according to whether they clotted milk or failed to do so. The failure of *B. bulgaricus* to ferment maltose made it possible for the writer (1915) to demonstrate that this organism apparently does not exist as an intestinal inhabitant in human beings. The examination of hundreds of strains of aciduric bacilli from human feces that did not embody purposely ingested *B. bulgaricus* has failed to reveal a single organism of that type.

Rogers and Davis (1912) quote Hastings (1908), Hastings and Hammer (1909) and Heinemann and Hefferan (1909) as having shown that the Bulgarian bacillus is "widely distributed and may be isolated from almost any sample of milk." Most of these authors admit the difficulty of cultivating aciduric bacteria in glucose broth, and this is true if the "natural" acidity of the broth is interfered with. The unneutralized broth mentioned above overcomes this difficulty, probably owing to its unaltered amino acids or other growth-promoting substances, such as were described for the meningococcus by Lloyd (1917). In the present investigation organisms corresponding to the

Bacillus bulgaricus were isolated from milk in but two instances, fourteen other strains proving to be aciduric organisms of another type.

The *Bacillus acidophil-aerogenes* of Torrey and Rahe (1915) is an aciduric organism differing from the bacillus of Moro in that it forms gas. The *Bacillus bifidus* is easily identified upon morphological grounds. Opinion as to its anaerobic requirements has lately undergone modification (Howe, 1917). We know that the characters just mentioned differentiate the organisms in some fashion, but when as groups, and when as individual bacilli, has never been determined. The object of the present investigation is to determine whether a better classification might not be made.

The methods employed in this work were, with some modification, those used by the writer in his earlier investigations in this field. The selective medium was acetic acid-glucose-broth having an acidity of N/20. This was seeded with the material under examination and incubated for three days. The method of triple seeding employed by Kendall (1910) was early abandoned, confirmation of the aciduric nature of the organisms being obtained by primary cultivation in this broth of the colonies fished from agar plates. Unneutralized glucose-oleate agar was superseded by the glucose liver agar of Torrey (1917). This medium has a reaction of plus 3.

In the cultivation of the bacteria of the type of *B. acidophilus* incubation for two or three days is desirable, though this is not always necessary. All cultures were carried in the unneutralized meat-peptone-glucose broth, and, with the various test substances substituted for glucose, this broth was used in the cultural tests. The *Bacillus bifidus* was grown in 0.5 per cent glucose agar having an acidity of plus 2. The strains of this organisms were isolated in part from human and in part from canine stools. The new differential plating method devised by Torrey (1917) was employed for this purpose.

It will be seen upon reference to the table that the cultures examined were obtained from a variety of sources. In saliva the *Bacillus acidophilus* and the *Bacillus acidophil-aerogenes*

occurred with equal frequency, while the former bacillus was not so frequently encountered in house sewage or milk. In human and animal feces as well as in saliva there is at times a tendency towards the appearance of one species to the exclusion of the other. Stomach contents from five cases of gastric carcinoma were examined and both types of organisms isolated, thus disposing of the Boas-Oppler bacillus as a separate entity. None of the carcinoma strains corresponded to *B. bulgaricus*, differing in this from some strains isolated by Heinemann (1917).

The classification herein suggested is based on fermentation tests. Among the aciduric bacilli, other than *B. bifidus*; colony formation, morphology and staining properties are subject to variation common to all. In the case of *B. bulgaricus*, action on milk and possibly colony formation assume the importance of distinct characters.

BACILLUS ACIDOPHILUS

As table 1 shows, it would be possible, on the basis of fermentation tests to define six subtypes of *B. acidophilus*. Mannite proved both here and elsewhere to be a very unsatisfactory test substance, permitting a very meager development at best. If we regard this alcohol as without value in differentiation the number of types becomes reduced to four. It is evident that the great majority of these organisms were milk clotters, thus agreeing with the *B. acidophilus* of Moro. The organism of Finkelstein does not possess this property. I am unable to account for the rarity of the non-clotting bacillus in this instance. In a previous investigation it was encountered with much greater frequency (Rahe 1914).

B. ACIDOPHIL-AEROGENES

The variable behavior of this organism in milk was noticed in the paper in which it was originally described (Torrey and Rahe 1915). The fermentation tests show a greater unity here than was the case in the preceding group. Sixty-five per cent of the cultures produced gas in all of the test substances if we except

TABLE 1

Non-gas producing aciduric bacilli (B. acidophilus)

CULTURE	SOURCE	COLONY TYPE	GROWTH IN UNNEUTRALIZED GLUCOSE BROTH	MILK-TIME OF CLOTTING	INCREASE ACIDITY GLUCOSE BROTH 5 DAYS	CULTURAL	B. ACIDOPHILUS
Sa- 7	Saliva	I	Good	24 hours			
Sa-18	Saliva	I	Good	24 hours	11.7	A_1Fermented	
Sa-23	Saliva	I	Heavy	48 hours	4.2	Glucose	
Sa-24	Saliva	I	Heavy	48 hours	7.1	Maltose	
Sa-25	Saliva	I	Heavy	18 hours	11.7	Raffinose	
Sa-26	Saliva	I	Heavy	24 hours	12.3	Sucrose	
Sa-27	Saliva	I	Heavy	48 hours	7.0	Lactose	
Sa-22	Saliva	I	Good	48 hours	12.2	Mannite	
506	Feces	I	Heavy	24 hours	7.3		
G	Feces	I	Heavy	24 hours			
D G C	Feces	I	Heavy	24 hours			
M-1	Milk	I	Good	18 hours			
M-4	Milk	I	Fair	18 hours			A
S-1	Carcinoma	I	Good	48 hours	7.2		
S-8	Carcinoma	I	Good	48 hours	13.7		
G-2	Feces	I	Good	Not clotted	7.9		
Har	Feces	I	Good	Not clotted	4.6	A_2Fermented	
Sa-3	Saliva	I	Good	24 hours		Glucose	
Sa-6	Saliva	I	Fair	48 hours		Maltose	
Sa-5	Saliva	I	Heavy	48 hours		Raffinose	
Sa-10	Saliva	I	Fair	48 hours		Sucrose	
515	Dog feces	I	Good	18 hours	11.3	Lactose	
D	Feces	I	Good	Not clotted	6.6		
M 11	Milk	I	Heavy	18 hours	8.7		
Sa-21	Saliva	I	Heavy	48 hours	12.6	B_1Fermented	
						Glucose	
507	Feces	I	Heavy	24 hours	8.0	Maltose	
K	Feces	I	Heavy	24 hours	12.2	Sucrose	
M 10	Milk	I	Heavy	18 hours	11.1	Lactose	
S_2	Carcinoma	I	Heavy	5 days	7.2	Mannite	
Ber. C	Feces	I	Heavy	48 hours		B_2Fermented	B
Sa-1	Saliva	I	Good	48 hours		Glucose	
Sa-2	Saliva	I	Good	72 hours		Maltose	
Sa-11	Saliva	I	Fair	24 hours		Sucrose	
502	Sewage	I	Good	6 days	13.0	Lactose	
M 5	Milk	II	Good	18 hours			
						Fermented	
						Glucose	C
1-a	Feces	I	Good	Not clotted		Maltose	
B	Feces	I	Good	Not clotted		Sucrose	
Sa-6	Saliva	I	Fair	48 hours		Fermented	
						Glucose	D
						Maltose	

TABLE 2

B. acidophil-aerogenes

CULTURE	SOURCE	COLONY TYPE	GROWTH IN UNNEUTRALIZED GLUCOSE BROTH	MILK-TIME OF CLOTTING	INCREASE IN ACIDITY GLUCOSE BROTH 5 DAYS	CULTURAL	B. ACIDOPHIL-AEROGENES
Sa-19	Saliva	I	Heavy	48 hours	8.3	Fermented (gas)	
Sa-20	Saliva	I	Heavy	7 days	10.3	Glucose	
H-1	Feces (Hen)	I	Heavy	15 days		Maltose	
Sa-32	Saliva	I	Heavy	72 hours	9.3	Raffinose	
Sa-33	Saliva	I	Heavy	72 hours	11.3	Sucrose	
Sa-34	Saliva	I	Good	72 hours	8.0	Lactose	
Sa-35	Saliva	I	Good	24 hours	7.7		
Sa-36	Saliva	I	Good	5 days	13.1		
Berd	Feces	I	Fair	8 days			
501	Sewage	I	Good	10 days	11.5		
511	Sewage	I	Heavy	48 hours	11.2		
F	Feces	I	Heavy	Not clotted			
C-2	Feces	I	Heavy	Not clotted	8.0		
Baby 1	Feces	I	Heavy	Not clotted	12.0		
Baby 2	Feces	I	Heavy	Not clotted	13.5		
Baby 3	Feces	I	Heavy	Not clotted	12.5		
Cs 4	Feces	I	Heavy	Not clotted	8.0		
Jam	Feces	I	Heavy	10 days	10.5		
Buck	Feces	I	Heavy	72 hours	9.5		
C-6	Feces	I	Heavy	Not clotted	8.0		
C-5	Feces	I	Heavy	Not clotted	8.5		
H	Feces	I	Heavy	Not clotted	10.5		A
C-1	Feces	I	Heavy	Not clotted	9.0		
Case 3	Feces	I	Heavy	Not clotted	9.3		
Bel-9	Feces	I	Heavy	7 days			
X-1	Feces (hen)	I	Heavy	21 days			
D-7a	Feces (dog)	II	Heavy	18 hrs.			
D-7b	Feces (dog)	I	Heavy	9 days			
D-8a	Feces (dog)	II	Heavy	8 days	5.2		
D-8b	Feces (dog)	II	Fair	8 days	6.3		
D-8c	Feces (dog)	II	Heavy	7 days	6.2		
R-8	Stomach carcinoma	II	Heavy	48 hours	4.5		
R-13	Stomach carcinoma	I	Heavy	7 days	6.8		
L-1	Stomach carcinoma	I	Heavy	15 hours	14.5		
S-2	Stomach carcinoma	II	Heavy	18 hours	7.4		
Ro-1	Stomach carcinoma	I	Heavy	Not clotted	8.3		
L-3	Stomach carcinoma	I	Heavy	Not clotted	8.0		
505	Sewage	I	Heavy	6 days	7.3	Growth without gas in raffinose	B
509	Feces (monkey)	II	Heavy	48 hours	8.3		

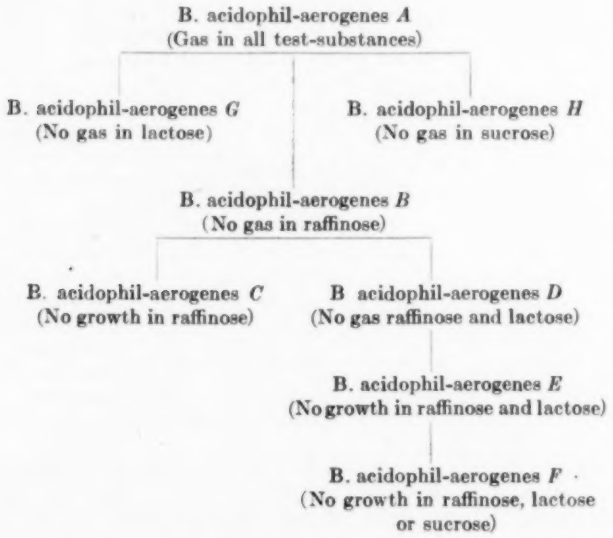
TABLE 2—*Continued*

CULTURE	SOURCE	COLONY TYPE	GROWTH IN UNNEUTRALIZED GLUCOSE BROTH	MILK-TIME OF CLOTTING	INCREASE IN ACIDITY GLUCOSE BROTH 5 DAYS	CULTURAL	B. ACIDOPHILI-AEROGENES
C-3	Feces	I	Heavy	Not clotted		Gas in	B
D-1	Feces (dog)	I	Heavy	15 days		Glucose	
M-12	Milk	II	Good	6 days	4.9	Maltose	
M-16	Milk	II	Good	18 hours	11.1	Sucrose	
S-7	Stomach carcinoma	I	Good	48 hours		Lactose	
						No growth in raffinose	C
M-14	Milk	II	Good	12 days	5.0	Gas in	
M-15	Milk	II	Good	6 days	4.0	Glucose	
						Maltose	
						Sucrose	
						Lactose	
						Growth without gas in lactose and raffinose	D
Sa-3	Saliva	I	Fair	24 hours		Gas in	
503	Sewage	I	Heavy	Not clotted	10.0	Glucose	
M-17	Milk	II	Good	8 days		Maltose	
						Sucrose	
						No growth in lactose and raffinose	E
Sa-16	Saliva	I	Good	Not clotted	6.5	Gas in	
504	Sewage	I	Heavy	Not clotted	10.0	Glucose	
						Maltose	
						Sucrose	
						No growth in lactose, raffinose and sucrose	F
Sa-15	Saliva	I	Fair	48 hours	9.0	Gas in	
						Glucose	
						Maltose	
						Growth without gas in lactose	G
Sa-28	Saliva	I	Good	Not clotted	8.2	Gas in	
Sa-29	Saliva	II	Good	Not clotted	6.4	Glucose	
500	Sewage	I	Good	14 days	6.5	Maltose	
M-2	Milk	I	Good	18 hours		Raffinose	
D-3	Feces (dog)	I	Heavy	48 hours		Sucrose	

TABLE 2—*Concluded*

CULTURE	SOURCE	COLONY TYPE	GROWTH IN UNNEUTRALIZED GLUCOSE BROTH	MILK-TIME OF CLOTTING	INCREASE IN ACIDITY GLUCOSE BROTH 5 DAYS	CULTURAL	B. ACIDOPHIL-AEROGENES
Ro. 1	Stomach carcinoma	I	Good	12 days	4.7	Growth without gas in sucrose Gas in	
508	Feces (monkey)	I	Good	Not clotted	4.7	Glucose Maltose Raffinose Lactose	H

mannite. The disparity in numbers between the bacillus A and the next most numerous member of this group hints that we may be dealing with a main type and its variants. Plausibility is given to this suspicion by the progressive nature of the assumed lapses in character. The diagram below will illustrate this point.



The diagram represents observed "lapses" only. More extended observations are necessary if the sequence is to be correct. There might be found, for example, the bacillus intermediate between *E* and *F*; i.e., one that grows in sucrose but does not produce gas and does not grow in raffinose and lactose.

Although the failure of *B. acidophil-aerogenes* first, to produce gas in a given carbohydrate and then to ferment it entirely, is suggestive in view of the numerical relationships of the different strains, we are not at liberty to conclude absolutely that such failures are instances of suppressed function and not real cultural differences. The cultural differences shown in the table are those found in freshly isolated strains and were not brought about intentionally. Prolonged artificial cultivation or other means might bring about such alterations, but until these are observed actually to occur it is better to assume that these differences are absolute and classify the organisms accordingly.

BACILLUS BULGARICUS

In table 3 are the organisms that correspond to the *Bacillus bulgaricus*. Some of these strains were obtained from the American Museum of Natural History while others were from commercial preparations of one kind or another. One strain was isolated from the saliva of a man, who, as far as could be determined, had not recently taken this bacillus. Two strains came from grade B. milk and made it appear that *B. bulgaricus*, in common with other lactic acid bacteria is able to survive the temperature of pasteurization. Experiments made to test the heat resisting properties of *B. acidophilus*, *B. acidophil-aerogenes* and *B. bulgaricus* showed them to be capable, in twenty-four hour broth culture, of surviving moist heat for one hour at 65°C.

Examination of the table shows that there is a very fair degree of uniformity between the various members of this group, both as regards colony formation and action on milk, fifteen out of seventeen strains showing close resemblance in this respect. Cultural tests permit their separation into four types. White and Avery (1910) recognized two types of *B. bulgaricus*. Their type A consisted of bacilli that were rapid fermenters, forming inactive lactic acid and exhibiting no granules, while type B showed granules, was less active and formed levo-rotary acid. These characteristics are not exclusive properties of *B. bulgaricus*.

TABLE 3

B. bulgaricus

CULTURE	SOURCE	COLONY TYPE	GROWTH IN UNNEUTRALIZED GLUCOSE BROTH	MILK, TIME OF CLOTTING	MILK, ACIDITY IN 6 DAYS	CULTURAL	
1	Museum	II	Good	18 hours	26.3	Fermented Glucose and Lactose	A
2	Fairchild	II	Good	18 hours	25.3		
3	Museum	II	Good	24 hours	26.5		
5	Museum	II	Good	18 hours	20.9		
6	Fermented milk	II	Good	18 hours	13.0		
7	Fermented milk	II	Good	24 hours	14.8		
8	Fermented milk	II	Good	24 hours	14.4		
9	B. B.	II	Good	24 hours	25.4		
11	Tablet	II	Good	24 hours	28.0		
M 7	Grade B milk	II	Good	18 hours	25.0	Fermented	B
M 8	Grade B milk	II	Good	18 hours	26.0	Glucose, Lactose and Raffinose	
B^1	B. B.	II	Good	48 hours	24.0		
B_2	Fairchild	II	Good	18 hours	26.0		
10	Massolin	II	Good	24 hours	11.4	Fermented	C
12	Tablet	II	Good	24 hours	24.6	Glucose, Lactose and Sucrose	
Sa8	Saliva	I	Good	48 hours			
4	Museum	II		18 hours	25.7	Fermented Glucose, Lactose, Sucrose and Raffinose	D

BACILLUS BIFIDUS

This organism is usually classed with the aciduric bacteria, presumably because of its occurrence in infant feces. That the bacillus is truly aciduric was shown by tests in which various strains were grown anaerobically in N/20 acetic-acid-glucose-

broth, with and without the addition of blood. Grown in unneutralized glucose broth, some strains produced an acidity of plus seven after five days incubation. I was unable to detect gas even when blood was added to the medium. Anaerobic milk cultures gave irregular results. Some cultures clotted the milk in periods ranging from eight to fifteen days, giving rise to an acidity that did not go beyond plus 7. In other instances the organisms failed to grow.

The figures in the table below represent the increase in acidity in puncture cultures in 0.5 per cent agar containing 1 per cent of the various test substances. The incubation period was five days.

TABLE 4

B. bifidus. Increase in acidity in 0.5 per cent agar after five days at 37°

CULTURE	SOURCE	MALTOSE	SUCROSE	LACTOSE	RAFFINOSE	GLUCOSE	MANNITE
D-7	Dog feces	3.3	6.0	4.1	3.4	3.7	0.0
D-1	Dog feces	7.1	5.0	2.4	4.6	2.4	0.0
C.	Human feces	4.0	2.1	3.2	2.9	3.4	0.0
D-3	Dog feces	0.8	0.7	1.9	1.9	2.5	0.0
D-9	Dog feces	3.6	2.6	5.4	1.2	6.6	0.0
D-6	Dog feces	3.5	1.8	3.1	2.6	3.6	0.4
Saun	Human feces	5.1	3.4	5.3	4.9	4.0	0.0
Pup	Puppy feces	13.3	5.0	11.9	9.6	9.8	2.2
Nor.	Human feces	5.7	2.5	4.6	5.1	4.0	0.5
D-11	Dog feces	8.3	6.4	9.0	9.3	9.8	2.4
D-1a	Dog feces	11.1	8.3	12.2	10.1	9.1	1.1
D-9a	Dog feces	+	+	+	+	+	+
D-9b	Dog feces	+	+	+	+	+	+
McE.	Human feces	+	+	+	+	+	+

In the latter part of this experiment the process of sugar freeing the broth used in the preparation of the medium was carried beyond the usual stage to determine whether the products resulting from the greater development of *B. coli* served to enrich the agar. Table 5 shows the results of comparative tests of half per cent agar, one lot of which was subjected to more extensive proteolysis by *B. coli* than the other. A distinct cloudiness accompanied the increased acid production. It is evident that the earlier cessation of growth in some lots of medium is due,

not to the accumulation of acid products of growth, but rather to the exhaustion of essential amino acids or other growth promoting substances.

Aside from their action on mannite and milk all of the strains of *B. bifidus* acted uniformly, and if we limit ourselves to the easily applied cultural tests in 0.5 per cent agar we find but two possible types of this organism. Even that arrangement would require defense, since the action on mannite was always slight. While the nature of *B. bifidus* is not yet entirely settled, Noguchi's (1910) demonstration of its pleobiosis has lately been confirmed

TABLE 5

Showing the greater activity of B. bifidus in medium 2, prepared from broth which had previously been subjected to the more prolonged action of B. coli

CULTURE	SOURCE	MALTOSE	SUCROSE	LACTOSE	RAFFINOSE	GLUCOSE	MANNITE	MEDIUM LOT
Pup	Puppy feces	2.8	3.4	3.1	2.6	3.7	0.5	1
Pup	Puppy feces	13.3	5.0	11.9	9.6	9.8	2.2	2
D 11	Dog feces	4.6	3.1	3.6	3.4	5.8	0.7	1
D 11	Dog feces	8.3	6.4	9.0	9.3	9.8	2.4	2
D 1a	Dog feces	2.9	1.6	4.5	0.4	4.9	0.4	1
D 1a	Dog feces	11.1	8.3	12.2	10.1	9.1	1.1	2

by Howe (1917), and in view of its protean nature, we must keep in mind that we are here dealing with the aciduric phase of this organism only. In its aciduric phase, then, the *Bacillus bifidus* is a non-gasforming organism that may or may not clot milk but ferments maltose, glucose, lactose, sucrose and raffinose.

The figures shown on opposite page illustrate the relative frequency with which the individual members of each of three of the groups of aciduric bacilli were found during the course of the present investigation.

It is unfortunate that, except in case of *B. bulgaricus*, the action on milk could not be correlated with the other cul-

tural test. In the instance of organisms of the type of *B. acidophilus* 90 per cent of the strains clotted milk, while with *B. acidophil-aerogenes* this figure falls to 65.

There is, in recent bacteriological literature, an instance illustrative of the desirability for a definite classification of the aciduric

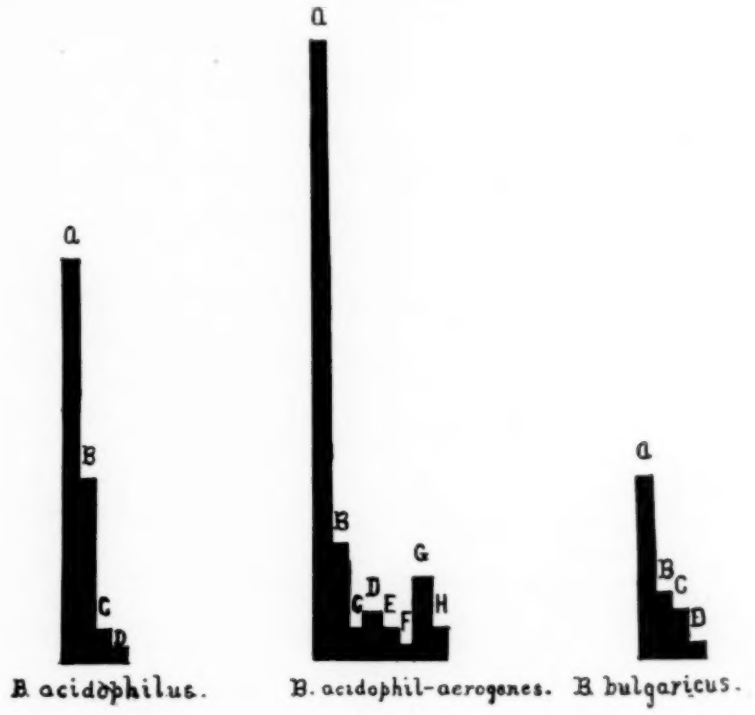


FIG. 1.

bacteria. Clark (1916), was unable to come to a definite conclusion as to the H ion concentration of the *Bacillus bulgaricus* owing in part to his uncertainty as to the identity of the particular organisms with which he was dealing. A classification, such as is given in the table below, would simplify investigations involving aciduric bacilli.

TABLE 6

Scheme for the classification of the aciduric bacilli including the aciduric phase of B. bifidus

BACILLUS		MALTOSE	GLUCOSE	LACTOSE	SUCROSE	RAFFINOSE	MILK
B. acidophilus	A	+	+	+	+	+	±
	B	+	+	+	+	−	±
	C	+	+	−	+	−	−?
	D	+	+	−	−	−	±
B. acidophil-aerogenes	A	g	g	g	g	g	±
	B	g	g	g	g	+	±
	C	g	g	g	g	−	±
	D	g	g	+	g	+	±
	E	g	g	−	g	−	−?
	F	g	g	−	−	−	+?
	G	g	g	+	g	g	±
	H	g	g	g	+	g	±
B. bulgaricus	A	−	+	+	−	−	+
	B	−	+	+	−	+	+
	C	−	+	+	+	−	+
	D	−	+	+	+	+	+
B. bifidus Aciduric phase		+	+	+	+	+	±

\+ = fermentation of carbohydrate or clotting of milk.
G = gas formation.
± = positive reaction in some instances negative in others.
− = no growth.

CONCLUSION

The aciduric bacilli, including *B. bifidus* in its aciduric phase, may best be classified in accordance with their abilities to ferment certain carbohydrates.

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THE GERMICIDAL ACTION OF FREEZING TEMPERATURES UPON BACTERIA

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Temperature is one of the cardinal factors influencing life activities of micro-organisms. The majority of bacteria are unable to exercise normal metabolism at temperatures below 6°C. or above 45°C.

Temperatures above the maximum are injurious to bacteria, and any appreciable increase above this critical point leads to death. Death of bacteria, due to high temperature, is not instantaneous, but proceeds in an orderly fashion at a rate which is predictable, though very rapid increases in the degree of heat may accelerate death so that it appears immediate. This has been explained as the outcome of accelerated metabolism which leads to auto-destruction. The destructive influence of other disinfectants, both physical and chemical, proceeds in a similar manner and may have a similar explanation.

Cold as a disinfectant seems to be an exception to this rule; in fact it has the opposite effect, as metabolism is arrested to its lowest ebb by temperatures lower than the minimum, and just below this critical point survival should, theoretically, be at its maximum. When the temperature is depressed to the freezing point or below, new factors enter into the problem and it is with these that this paper is primarily concerned.

The influence of low temperatures on micro-organisms has received relatively little attention. In 1882 Pumpelly found that samples of ice cut from the center of the block and inoculated into sterile beef broth showed living contamination.

The first extensive investigations were made by Prudden in 1887. Pure cultures of *B. prodigiosus* and *B. proteus* were found to be sterile after fifty-one days at temperatures ranging between $-10°$ to 1°C. *B. typhosus* survived for at least one hundred and three days at a temperature between 14° and 30°F. It will be of interest in relation to the data presented later to reproduce the results obtained by alternate freezing and thawing.

By coating tubes with sweet oil to prevent the crystallization of the water, and comparing the death rates in these tubes with

TABLE 1

*Continuous freezing compared with alternate freezing and thawing**

FROZEN SOLID		ALTERNATE FREEZING	
B. typhosus			
Before freezing	40,896	Before freezing	40,896
Frozen 24 hours	29,780	Frozen 3 times	90
Frozen 3 days	1,800	Frozen 5 times	0
Frozen 4 days	950	Frozen 6 times	0
Frozen 5 days	2,490		
B. prodigiosus			
Before freezing	339,516	Before freezing	339,516
Frozen 24 hours	36,410	Refrozen 1 time	2,570
Frozen 30 hours	41,580	Refrozen 2 times	275
Frozen 48 hours	14,440	Refrozen 3 times	15
Frozen 96 hours	4,850	Refrozen 4 times	0

* Frozen at 2° to 5° F., then kept just below 32° F.

those in which solid freezing had occurred, the former showed greater fatality. Prudden concludes that "the greatest reduction occurs during, or shortly after the sudden reduction of temperature to freezing, and, if after this the bacteria remain in ice, a comparatively gradual destruction goes on; if bacteria are thawed out and immediately refrozen another large increment is destroyed." The killing action of cold as such is chiefly emphasized.

Ravenel, (1899) Macfayden, and others have obtained very low temperatures with liquid air and liquid hydrogen (-252)

and have concluded in general that cold cannot be depended upon to sterilize.

Park (1901) records 100 per cent reduction with twenty different strains of typhoid subjected to −5°C. for twenty-two weeks. From their extensive work Sedgwick and Winslow (1902) concluded that not only different species but different "races" within the same species exhibit marked variability in their resistance to freezing temperatures.

Keith, (1913) contrary to Prudden, has emphasized the importance of solid freezing as compared with cold in relation to the death rate of bacteria. *B. coli* frozen solidly in water at −20°C. show 99 per cent killed in five days, but when not actually frozen a large per cent remain alive for months; when frozen in diluted milk the death rate increases with the dilution; when suspended in aqueous mixtures of 5 to 42 per cent glycerine a large percentage remain alive for six months at −20°C. Keith concluded that the important factors influencing the death rate of bacteria at low temperatures are their rate of metabolism and the mechanical protection offered by the medium.

In 1915 one of the authors in collaboration with Torossian and Stone published notes on the germicidal effect of freezing and low temperatures. We suggested there that "bacteria may be killed by the mere fact of low temperature, interfering with metabolism; by freezing of the cell contents and rupture of the membrane by internal pressure; by external pressure or grinding developed during crystallization; or by expansion of the frozen medium within the receptacle; or by more or less prolonged suspension of metabolic activities, leading to slow death from old age or starvation." Below we reproduce the tables as given in these notes. The extension of the work since this time has entirely confirmed this preliminary study as regards (a) the rapid destruction of 90 per cent or more of *B. coli* when frozen in tap water for three hours, (b) the greater viability of spores (*B. subtilis*) in frozen mixtures, (c) the greater germicidal influence of intermittent freezing and thawing, (d) the fact that depression of the temperature within certain limits

increases slightly the germicidal activity, (e) and the fact that cream and milk furnish some protection to bacteria frozen in them either continuously or intermittently.

We were most interested to learn, if possible, whether some external factor, other than the low temperature, entered into the

TABLE 2

A comparison of the percentage reduction of B. coli held at 0.5°C., −15°C., and frozen intermittently in tap water for a three hour period

INITIAL COUNT	FIRST FREEZING	SECOND FREEZING	THIRD FREEZING	FOURTH FREEZING	CONTINUOUS FREEZING THREE HOURS −15°	COLD 0.5°C THREE HOURS
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
2,130	82.2	99.9	99.9	99.9	99.9	23.0
1,650	92.8	96.1	99.8	99.9	99.7	29.0
1,320	93.8	98.7	99.9	99.9	99.4	47.0
3,015	97.6	99.6	99.5	99.9	99.8	31.3
4,800	98.6	99.4	99.8	99.8	99.8	32.0
1,370	98.6	99.5	99.8	99.9	99.7	8.1
1,070	97.9	99.5	99.5	99.9	99.9	97.3

TABLE 3

Percentage reduction obtained with B. coli in cream at freezing temperatures

INITIAL COUNT	FIRST FREEZING	SECOND FREEZING	THIRD FREEZING	FOURTH FREEZING	THREE HOURS FREEZING 0°C.	COLD 0.5°C. THREE HOURS
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
4,350	4.8	39.3	45.0	48.9	61.3	18.0
4,740	40.5	45.5	71.5	75.9	67.7	42.7
5,275	43.1	46.7	71.9	81.2	44.2	16.4
5,284	33.4	48.2	60.2		26.4	20.8
5,028	32.2	36.2	48.4	71.7	34.8	20.6
3,732	35.2	20.9	42.3	50.1	33.6	38.9
4,030	71.0	67.1	78.6	83.1	67.6	3.9
5,085	21.1	51.6	53.3	75.4	65.3	23.8
4,725	16.1	36.5	52.2	72.6	58.0	16.8
4,560	34.8	47.1	67.4	63.8	54.2	19.7

destruction of the bacteria. Is there a critical degree of cold at, or, just below, freezing which is highly fatal, or is the crystallizing action itself destructive? We have noted the conclusion of Prudden, where cold itself was chiefly emphasized. Keith emphasized the solidification. Most work on cold has been

tested over a long period of time without relation to the quantitative aspect, while we have studied chiefly its influence over very short periods and have consistently made quantitative studies.

In order to eliminate crystallization and possible mechanical crushing of bacteria during the freezing of the medium, the freezing point was depressed by the addition of a non-electrolytic substance, grape sugar. From the formula given in Harper's Scientific Memoirs for the lowering of the freezing point of water in degrees Centigrade, produced by dissolving a gram molecule of a given substance in a liter of water, it was possible to

TABLE 4

	GLUCOSE
	grams
To depress the freezing point to −6°C.	56.2
To depress the freezing point to −4°C.	37.5
To depress the freezing point to −3°C.	28.1
To depress the freezing point to −2°C.	18.7
To depress the freezing point to −1.5°C.	14.0
To depress the freezing point to −0.5°C.	4.7

calculate the amount of glucose ($C_6H_{12}O_6$) which must be added to a liter of water to depress its freezing point by any desired increment. The table above gives the amount of glucose required to depress the freezing point to a certain degree as worked out by the above formula.

The several sugar solutions were made with chemically pure glucose and sterilized in streaming steam on three successive days. The pure cultures of *B. coli*—six strains in all were used—were frequently transferred and a twenty-four hour old culture in standard bouillon was always used in the tests. In all of the experiments sterile tap water was used as a control, and for comparison. The culture was inoculated into the tubes of water and solutions to be tested, the initial numbers determined by plating from each tube in several dilutions, always using duplicate plates, and then the tubes were immersed in an ice-

salt bath of a concentration to give approximately the temperature desired, and the temperature was watched for the period of the experiment and adjusted when necessary. Melting of frozen tubes was brought about gradually by immersion in cold water. All plates were incubated at 37°C. and counted after forty-two hours.

To determine that the glucose in the concentrations used was not germicidal, either on account of its chemical or osmotic properties, each solution was inoculated and placed in the refrigerator at from 4 to 6°C. for three hours. At least four tests for

TABLE 5

CONCENTRATION OF SUGAR	TEMPERATURE	SUGAR			WATER		
		Initial count	Final count	Reduction	Initial count	Final count	Reduction
grams per liter	*deg. C.*			*per cent*			*per cent*
4.7	−0.5	880	515	41.4	790	150	81.0
		613	378	38.3	682	109	85.4
		132	55	58.3	55	0	100.0
		650	300	53.8	435	6	98.6
		414	226	45.4	575	10	98.2
		150	88	41.3	1,340	18	98.6
			Average	49.6		Average	90.3
14.0	−1.5		Average	36.2		Average	95.9
18.7	−2.0		Average	40.8		Average	93.1
28.1	−3.0		Average	44.2		Average	96.9
37.5	−4.0		Average	49.3		Average	98.8
56.2	−6.0		Average	49.5		Average	99.2

each solution were made and the control in tap water was made each time. The reductions in sugar solutions were variable but were uniformly lower than those which occurred in water, so we feel entirely justified in concluding that the sugar as such had no germicidal influence.

We then undertook to compare the effect of low temperatures upon bacteria in a medium which crystallized, with one where crystallization was absent. The detailed data are given for the series of experiments at one temperature only and the average reduction of a similar series of experiments at each of the other temperatures is given in table 5.

Comparing the percentage reduction which occurs in the fluid sugar solutions, and the solidified water kept at the same temperature for the same period of time, and with all other factors as nearly identical as possible, it is readily seen that the death-rate is much higher in the solidified tubes. This indicates a very conspicuous rôle played by crystallization as such, regardless of the factor of cold.

This was tested still farther by actually freezing the strongest sugar solution to make certain that the solute itself did not somehow fortify against cold. Some of the results are given in table 6.

In this table the reductions obtained in the solidly frozen sugar solutions are uniformly higher than those obtained in the

TABLE 6

B. coli frozen solid at −10°C. for three hours in a solution of glucose 56 grams per liter and in tap water

SUGAR			WATER		
Initial count	Final count	Reduction	Initial count	Final count	Reduction
		per cent			*per cent*
633	50	92.1	55	1	98.1
855	8	99.0	318	1	99.6
2,900	32	98.8	1,340	2	99.8
7,560	1,700	77.5	5,770	87	98.4

previous results and are quite comparable with the reductions in the water controls.

From the foregoing discussions and data we venture to draw certain conclusions, appreciating, however, that the work is not extensive enough to render any of these statements final.

1. Intermittent freezing of bacteria exerts a more effective germicidal action than continuous freezing.

2. The reduction is much less in milk and cream than in pure tap water when freezing temperatures are applied, due, no doubt, to physical protection offered to the bacteria by the colloidal and solid matter in suspension.

3. The degree of cold below freezing is not a very important factor in the destruction of bacteria. There is no critical

temperature below freezing where the germicidal effect is greatly accelerated.

4. The death-rate of *B. coli* is much higher in media which are frozen solid than it is in the same media not solid and at a slightly lower temperature.

5. Crystallization, probably resulting in mechanical crushing, is an important germicidal factor in causing the death of bacteria at zero degrees Centigrade and below. The greatest reduction occurs promptly upon freezing and refreezing, but is not caused so much by the sudden change in temperature as by this mechanical factor.

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